Immunology of Granulomatosis with Polyangiitis

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Declaration

I declare that I have personally prepared this thesis. The work described here is my own, carried out personally unless otherwise stated. All sources of information, including quotations, are acknowledged by means of reference.

Yuan Zhao

October 2012

Dedication

To my parents

献给我的爸爸妈妈

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Abstract

Granulomatosis with polyangiitis (GPA, formerly known as Wegener's granulomatosis) is a rare and sometimes fatal systemic autoimmune disease. Anti-neutrophil cytoplasmic antibodies (ANCAs) specific for proteinase 3 (PR3) are associated with GPA. However, the pathogenesis of GPA is not yet clear. Our aim was to investigate the local autoimmune response, circulating immune modulatory cells and cells expressing the immune suppressor molecules programmed death 1 (PD-1) and its ligands in GPA.

In mucosa from GPA patients, activated B cells were observed located alongside PR3 expressing cells and B cell survival factors BAFF and APRIL, which was produced by the granulomas and giant cells. B cells were proliferating and persistent in biopsies. However no evidence of B cell clones from the mucosal biopsies circulating in peripheral blood was observed in GPA.

An increased frequency of circulating T_{FH} cells and a reduced frequency of Treg cells was observed in peripheral blood from GPA patients on conventional therapies compared to healthy controls. No such difference was found in GPA patients treated with rituximab. The frequency of circulating T_{FH} and Treg cells was found to be inversely correlated in human peripheral blood.

No difference in the relative quantity of mRNA encoding PD-1 in lymphocytes and monocytes was found in GPA patients compared with healthy controls. Lower percentage of CD14+ monocytes expressing PD-1 was observed in GPA patients. Lower relative quantity of mRNA encoding PD-1 ligands PD-L1 and PD-L2 in T cells and monocytes was observed in GPA patients.

In conclusion, data in this thesis identifies activated B cells alongside auto-antigens and B cell survival factors in the mucosa in GPA. A negative correlation between T_{FH} and Treg cells is observed that implies the balance between T cell subsets and its B cell dependence are associated with disease activity in GPA. The deficiency of PD-L1 and PD-L2 mRNA in lymphocytes and monocytes may contribute to the pathogenesis of GPA.

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Abbreviations

aa	amino acids
Abs	antibodies
AF488	Alexa Fluor@ 488
Ags	antigens
AID	Activation-induced cyditine deaminase
ANCA	anti-neutrophil cytoplasmic antibody
APC	allophycocyanin
APE	apurinic/apyrimidinic endonucleases
APRIL	a proliferation-inducing ligand
AZA	anathioprine
BAFF	B-cell activating factor
BAFF-R	B-cell activating factor receptor
BCD GPA	B cell depleted GPA patients
Bcl-6	B-cell lymphoma 6
BCMA	B cell maturation antigen
BLyS	B Lymphocyte Stimulator
bp	base pairs
BVAS	Birmingham vasculitis activity score
C	cytosine
cANCA	cytoplasmic anti-neutrophil cytoplasmic antibody

CCL25	chemokine (C-C motif) ligand 25
CCL27	chemokine (C-C motif) ligand 27
CCL28	chemokine (C-C motif) ligand 28
CCR10	C-C chemokine receptor type 10
CCR9	C-C chemokine receptor type 9
CD127	cluster of differeniation 27
CD138	cluster of differeniation 138
CD14	cluster of differeniation 14
CD19	cluster of differeniation 19
CD20	cluster of differeniation 20
CD25	cluster of differeniation 25
CD28	cluster of differeniation 28
CD3	cluster of differeniation 3
CD4	cluster of differeniation 4
CD40L	CD40 ligand
CD68	cluster of differeniation 68
CD8	cluster of differeniation 8
cDNA	complementary DNA
CDRs	complementarity determining regions
CLA	cutaneous lymphocytes antigen
CNS	centre nerve system

cpm	counts per minute
CSR	class switch recombination
CTACK	cutaneous T-cell-attracting chemokine
CTLA-4	cytotoxic T-lymphocyte antigen 4
CXCL13	C-X-C motif chemokine 13
CXCR5	C-X-C chemokine receptor type 5
CYC	cyclophosphamide
DC	dendritic cell
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
DSBs	double strand breaks
EAE	experimental autoimmune encephalomyelitis
Fas-L	Fas ligand
FITC	fluorescein isothiocyanate
FMO	fluorescence minus one
FoxP3	forkhead box P3
FRs	framework regions
FSC	forward scatter
GALT	gut-associate lymphoid tissues
GC	germinal centres

GPA	granulomatosis with polyangiitis
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HC	healthy controls
HEV	high endothelial venule
HLA	human leukocyte antigen
HSPG	heparan sulphate proteoglycan
ICOS	inducible co-stimulator
IFN-β	interferon beta
Ig	immunoglobulin
IGH	immunoglobulin heavy chain
IGHD	immunoglobulin heavy chain diversity region
IGHJ	immunoglobulin heavy chain joining region
IGHV	immunoglobulin heavy chain variable region
IHC	immunohistochemistry
IL-2	interleukin-2
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
ISD	immune suppression drugs
ITIM	immunoreceptor tyrosine-based inhibitory motif
iTreg	inducible regulatory T cell
ITSM	immunoreceptor tyrosine-based switch motif

J segment	joining gene segment
Ki67	antigen KI-67
LPS	Lipopolysaccharide
MadCAM-1	mucosal vascular addressin cell adhesion molecule 1
MALT	mucosa-associated lymphoid tissue
MEC	mucosae-associated epithelial chemokine
MEK	MAPK/ERK kinase
MHC	major histocompatibility complex
MMF	mycofenolate mofetil
MPO	Myeloperoxidase
MRI	Magnetic resonance imaging
mRNA	messenger RNA
MTX	Methotrexate
MyD88	Myeloid differentiation primary response gene 88
nCT	nactive choleva toxin
N-D	N-nucleoties and diversity gene segment
neg	negative
NOD	Non-obese diabetic
nTreg	natural regulatory T cell
OFG	orofacial granulomatosis
PAMPs	pathogen-associated molecular patterns

pANCA	perinuclear anti-neutrophil cytoplasmic antibody
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PD-1	programmed death-1
PD-L1	programmed death-1 ligand 1
PD-L2	programmed death-1 ligand 2
PD-L*s*	programmed death-1 ligands
PE	phycoerythrin
PerCP-Cy5.5	peridinin chlorophyll protein-Cy5.5
PI3K	phophatidylinositol-3-kinase
PRRs	pattern recognition receptors
PR3	proteinase 3
pre-BCR	pre-B cell receptor
Pred	prednisolone
pre-TCR	pre-T cell receptor
PTEN	phosphatase and tensin homolog
RNA	ribonucleic acid
RSSs	recombination signal sequences
RT-PCR	real-time PCR
RTX	rituximab
S region	switch region

SHM	somatic hypermutation
SHP-1	tyrosine phosphatase 1
SHP-2	tyrosine phosphatase 2
SLE	systemic lupus erythematosus
SSC	side scatter
TACI	transmembrane activator-1 and calcium modulator and cyclophilin ligand-interactor
TALL-1	TNF- and APOL-related leukocyte expressed ligand
TdT	Terminal deoxynucleotidyl transferase
TECK	thymus-expressed chemokine
T_{FH}	T follicular helper cells
TGF-β	transforming growth factor beta
Th1	type 1 helper T cells
Th17	T helper 17 cells
Th2	type 2 helper T cells
TI	thymus-independent
TNFSF13	tumor necrosis factor ligand superfamily member 13
TRAF6	TNF receptor associated factor 6
Treg	regulatory T cells
U	uracil
UNG	uracil-DNA glycosylase

US	United States
USA	United States of America
V segment	variable gene segment
VCAM-1	vascular cell adhesion molecule 1
VDI	vasculitis damage index score
VDJ junction	variable, diversity and joining junction
VISTA	V-domain Ig suppressor of T cell activation
α4β7	α4β7 integrin

Chapter 1

Main Introduction

1.1 Granulomatosis with polyangiitis (GPA)

Granulomatosis with polyangiitis (GPA) was formerly known as Wegener's granulomatosis [1]. It is a chronic inflammatory granulomatous autoimmune disease characterized by upper and/or lower airways disease and glomerulonephritis [2-5]. It can affect all ethnicities with an equal male to female ratio and higher prevalence in Caucasians (more than 90%) [6, 7]. It is one of the most common systemic vasculitic diseases in the UK and the annual incidence of GPA in the UK has been reported to be 8.5 cases per million [8].

GPA is a part of a spectrum of vasculitic syndromes that affect small and medium-sized vessels. The clinical symptoms of GPA are variable and many organs can be affected, such as the upper airways, lungs, kidneys, ears, skin, gut and the central and peripheral nervous systems [3]. The condition may be life-threatening and 75% of patients with GPA will eventually develop renal involvement, for example rapidly progressive glomerulonephritis and necrotizing crescentic glomerulonephritis which, if not recognized and treated early, can lead to end-stage renal disease or death [2, 3]. More than 80% of GPA patients suffer from nasal symptoms, such as pain, stuffiness, nosebleeds, rhinitis nasal bridge tenderness and crusting [3, 9]. Some patients have distinctive nasal septal perforations and a saddle-nose deformity, as shown in figure 1.1.1.

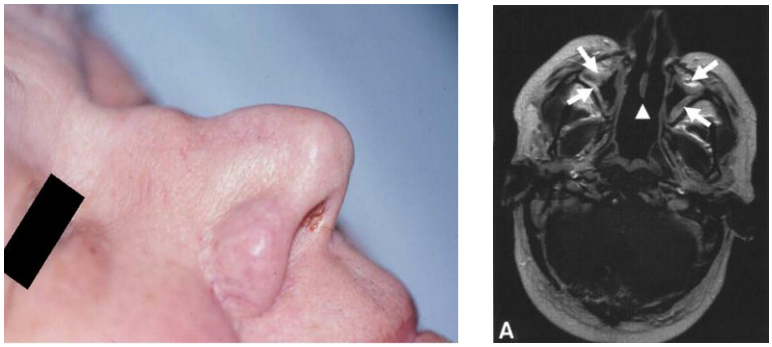

Figure 1.1.1 Saddle-nose deformity of GPA patient: left picture of an example of saddle-nose deformity in a patient with GPA [3]; **right** magnetic resonance imaging (MRI) of an example of saddle-nose deformity in a patient with GPA. The loss of nasal conchae and destruction of the nasal septum is indicated by triangle; the inflammatory tissue around is indicated by arrows [10].

GPA can also cause pulmonary manifestations such as pulmonary nodules, infiltrates, cavitary lesions and alveolar hemorrhage (Figure 1.1.2). Ear symptoms include ear pain, otitis externa and otitis media hearing loss. Other non-specific manifestations include oral mucosal ulcerations, skin lesions and arthralgia [3]. Central and especially peripheral nerve involvement with vasculitic neuropathies and peripheral nerve lesions can be disabling and there is a wide spectrum of ophthalmologic manifestations including orbital granulomas, scleritis, peripheral ulcerative keratitis, retinal vasculitis and visual loss [3].

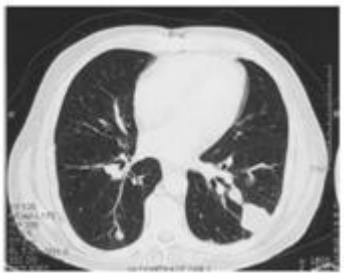

Figure 1.1.2 Multifocal cavitary nodules in lungs of patient with GPA [3]

To be classified as GPA, a patient has to fulfill a minimum of 2 of the 4 criteria: nasal or oral inflammation resulting in ulcers, purulent or bloody nasal discharge; chest radiographic abnormalities such as nodules, fixed pulmonary infiltrates or lung cavities; urinary sediment and granulomatous inflammation on tissue biopsies [11, 12]. These are not diagnostic criteria but are useful in classifying anti-neutorphil cytoplasmic antibody (ANCA) associated vasculitis as GPA. ANCA has been reported to have a specificity of 92% and a sensitivity of 88% in GPA diagnosis [13]. The diagnosis of GPA is primarily based on clinical manifestations of systemic vasculitis since some patients may be ANCA negative despite having clinical evidence of GPA. This small group of ANCA-negative GPA patients tend to have limited disease rather than severe systemic manifestations of GPA [14].

It is important to ascertain the level of disease activity in systemic vasculitis in order to devise the best treatment strategies for patients and monitor clinical response to therapy. The Birmingham Vasculitis Activity Score (BVAS) is a disease assessment instrument which provides a computer generated numerical score of vasculitis disease activity that can be applied directly to management of patients with GPA [15]. The extent of damage as a result of the granulomatous or vasculitic aspects of this disease can be measured annually with a vasculitis damage index score (VDI) [16]. The BVAS and VDI are instrumental in predicting possible disease outcome and prognosis.

If untreated severe GPA has a mortality rate at 1 year of approximately 80% with the major causes of death including severe necrotising glomerulonephritis, severe pulmonary haemorrhage and infection. The introduction of immunosuppressive therapy has greatly improved the morbidity and mortality of GPA patients and at present the 5 year survival rate is approximately 75% [17]. Induction and maintenance of remission in GPA may be achieved using standard immunosuppressive drugs. Cyclophosphamide (CYC) in combination with corticosteroids has been used via either as intravenous pulsed therapy or oral therapy in remission induction therapy for moderately-severe to severe manifestations of GPA [18]. Mild or localized GPA may be treated with Methotrexate (MTX) via the oral or subcutaneous route [19]. MTX and Azathioprine have also been reported to be effective for remission maintenance in GPA [20, 21]. Apart from standard immunosuppressive drugs, rituximab, an anti-CD20 monoclonal antibody, was licensed for the management of the ANCA-associated vasculitides, GPA and microscopic polyangiitis, by the United States Food and Drug Agency (FDA) in 2011 [22]. Rituximab has been shown to be effective in treating GPA patients (details described in section 1.9 and chapter 4) [23, 24].

In this thesis, various aspects of the immunology of GPA have been investigated. Before introducing what is known about the immunology of GPA from the literature, the relevant aspects of basic immunology will be described.

1.2 B cell maturation and Antibody production

1.2.1. Overview of B cell development

B cells producing antibodies (Abs) against antigen (Ag) play an important role in the humoral immune response of adaptive immune system [25]. They originate from stem cells in bone marrow [26]. In bone marrow, progenitor B cells go through immunoglobulin heavy chain gene rearrangement (pro-B cell stage) followed by light chain gene rearrangement (pre-B cell stage) and then become immature B cells [27, 28] (details described in section 1.2.2). CD20 is expressed on the surface of B cells from the late pro-B cell stage and is useful as a marker for B cells [28, 29]. The first checkpoint for autoreactive B cells occurs at this stage which only allows cells with low affinity to self Ags to survive [28, 30] (details described in section 1.2.3).

Immature B cells then leave bone marrow and enter peripheral blood. B cells at this stage are called transitional B cells [31]. It has been reported that there are three consecutive stages of transitional cells in human based on expression of transitional markers CD21, CD24, CD38, CD10, IgD and $CD45RB^{MEM55}$ [31, 32]. The second checkpoint for autoreactive B cells in which B cells expressing Abs against self Ags are deleted occurs at this stage though what contributes to this in humans is not known [33, 34]. B-cell activating factor (BAFF) has been reported to play an important role in B cell survival at this checkpoint [35] (details described in section 1.2.6).

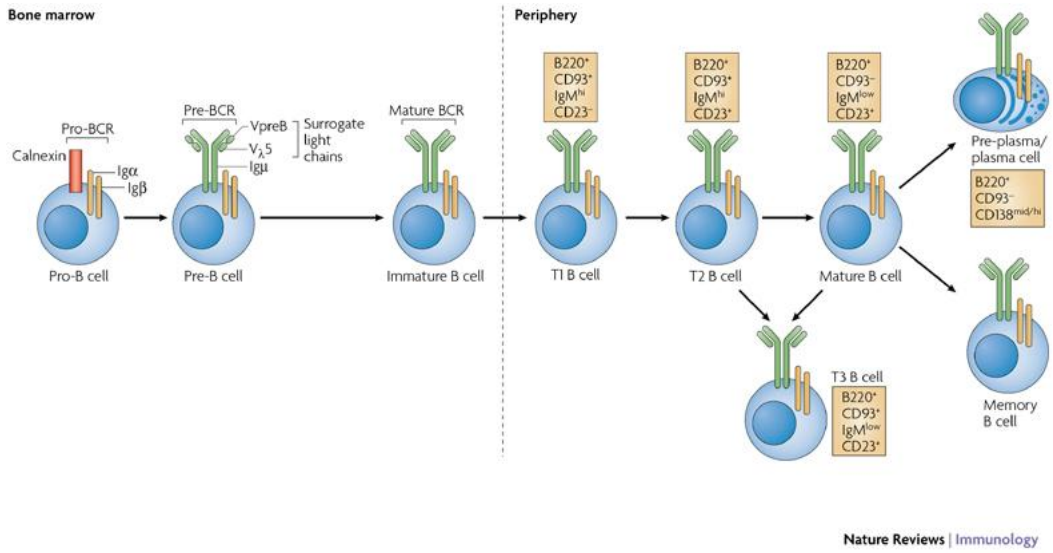

Figure 1.2.1 Overview of B cell development in bone marrow and periphery [36].

When the peripheral tolerance process is complete, B cells become fully mature and named naïve B cells [31]. They then recirculate through peripheral blood to secondary lymphoid tissues such as lymph nodes, spleen and Peyer's patches where they encounter Ags and may form germinal centres (GC) with help from activated T cells if they encounter specific Ag [37]. Activation of B cells resulting in GC formation requires the presence of Ag, interaction between CD40 on B cells and CD40ligand (CD40L) on T cells and cytokine stimulation [31, 38, 39]. The activated B cells then proliferate rapidly and form germinal centres where B cells undergo somatic hypermutation (SHM) and class switch recombination (CSR) to generate high affinity Abs and Ab diversity [40, 41] (details described in section 1.2.4). Activated B cells with low affinity to Ags are deleted while cells expressing high affinity Abs survive and leave GCs develop

into Ab-secreting plasma cells or memory B cells [42, 43]. CD20 is expressed on memory B cells, but is lost before terminal plasma cell differentiation [28, 29]. The overview of B cell development is shown in Figure 1.2.1.

Alternatively, B cells can be activated in a T cell independent manner. It has been suggested that the second signal provided by T cells in T cell dependent B cell response is replaced by either recognition of microbial constituents or cross-linking of BCRs by Ag with repeating epitopes [28] (details described in section 1.2.5).

1.2.2 Antibody production

1.2.2.1 Structure of Antibodies

The basic functional unit of antibody (Ab), also known as immunoglobulin (Ig), is a 'Y' shaped molecule which contains two identical antigen-binding sites at the tips of the arms of the 'Y'. Each Ab molecule contains two identical heavy chains and two identical light chains combined together by disulfide bonds. Both heavy and light chains are composed of two different functional domains: variable (V) domains encoding the antigen binding site and constant (C) domains determining different isotypes. There are five isotypes of Ig that differ in their functional properties in mammals: IgM, IgD, IgG, IgA and IgE. Flexible hinges in IgD, IgE and IgA connect the branch and stem together, while in IgM and IgE, there is an extra C region instead of hinge, as shown in Figure 1.2.2 [28].

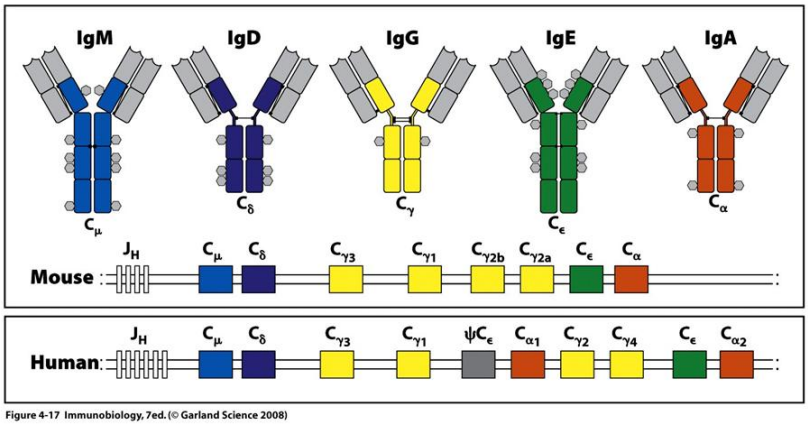

Figure 1.2.2 Different Ig isotypes are encoded by identical heavy chain C regions. IgD, IgG and IgA are encoded by three C_δ, C_γ and C_α gene segments respectively. IgM and IgE are encoded by four C_μ and C_ε segments respectively. An extra C region instead of the hinge combines the segments of heavy chain together [28].

The angle between two antigen-binding sites can be dramatically different according to the flexibility of the hinge, which enables Ab to recognize and bind to different antigens (Ags) [44]. Antigen-binding sites consist of V domains from both light chains and heavy chains. In the V domain of each polypeptipe chain, there are three segments having much higher variability termed complementarity determining regions (CDRs). CDRs determine binding ability to Ags. The regions between CDRs are termed framework regions (FRs) as they provide the structural framework of the antigen-binding site. There are three FRs in each germline V domain: FR1, FR2 and FR3, and an FR4 after the rearranged CDR3 [45].

1.2.2.2 Generation of Antibodies

Antibody diversity in the primary Ig repertoire is generated by gene rearrangement. Diversity by gene rearrangement can be combinatorial due to multiple alternative rearranging segments in germline or junctional because the joints between segments are not precise. Light chain consists of variable (V), joining (J) and constant (C) regions, while heavy chain has an extra diversity (D) segment between V and J segments. As many alternatives of V, D and J segments are present in germline DNA, approximately random selection of gene segment from each type makes V(D)J combination highly variable, and ensures the combinatorial diversity of gene rearrangement. Random deletion of nucleotides by exonuclease and addition of nucleotides in the joint of gene

segments in each chain guarantees junctional variability that also contributes to the diversity resulting from V(D)J recombination [28].

B cell development starts by rearrangement of Ig heavy chain loci [27, 46]. When gene rearrangement occurs in the heavy chain, one D segment combines randomly with one J segment by pairing of recombination signal sequences (RSSs) with opposite orientation and everything between is deleted. Then the joined DJ segments combine with one of the upstream V segments at random while nucleotides are added to the junction by Terminal deoxynucleotidyl transferase (TdT). After the VDJ junction is formed, the DNA strand is transcribed to primary transcript RNA which has an intron between VDJ junction and C region. Finally, messenger RNA (mRNA) is produced by splicing out the intron between VDJ junction and C segment [28]. B cells undergoing Ig heavy chain gene rearrangement at this stage are named pro-B cells [47].

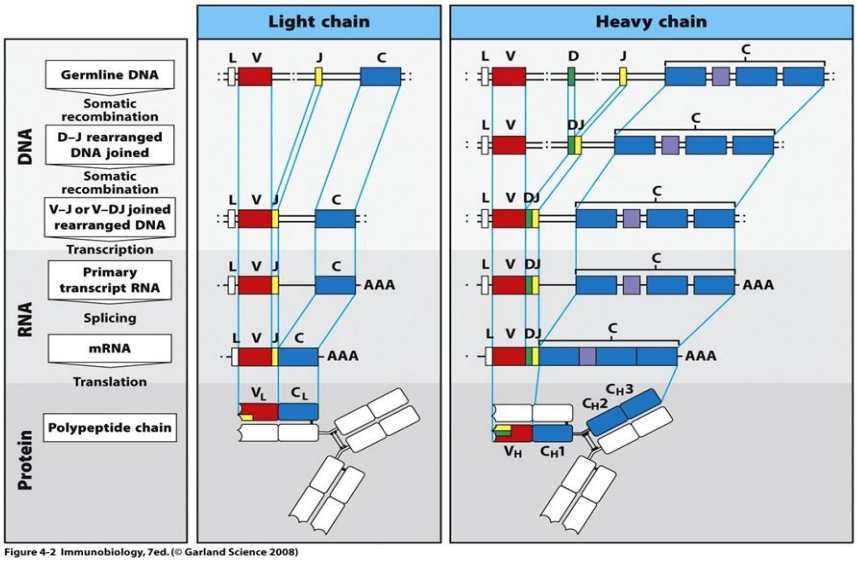

Figure 1.2.3 Gene rearrangement in heavy chain and light chain [28]: Ig heavy chain gene rearrangement (right) starts from random combination of one D segment with one J segment, then DJ junction combines with one randomly selected V segment. Gene sequences between the selected gene segments are deleted. Then template DNA is transcribed into mRNA containing an intron between VDJ junction and C segment which will be spliced out at the final stage**. Ig light chain gene rearrangement (left)** starts with random combination of one V segment with a J segment. DNA is then transcribed into mRNA which contains an intron between VJ junction and C segments. The intron is then spliced out when the light chain gene rearrangement is completed.

When the heavy chain rearrangement is completed, pro-B cells develop to pre-B cells at which stage Ig light chain gene rearrangement occurs [48, 49]. The process of VJ recombination in light chain is similar to that in heavy chain. As there is no D segment in light chain, rearrangement starts from the random combination of a V segment and a J segment, and the subsequent steps are the same as in heavy chain [28]. The sequence of Ig gene rearrangements is shown in Figure 1.2.3 [28].

1.2.3 Autoreactive B cells in early B cell development

It has been reported that the rearranged Ig heavy chain can be expressed on the surface of pre-B cells to form pre-B cell receptor (pre-BCR) [50]. At this stage, a Vpre-B and λ5 encoded surrogate protein replaces the conventional light chain by binding to rearranged heavy chain [51]. The expression of pre-BCR has been reported to play an important role in the transition from pro-B cell to pre-B cell. First checkpoint for autoreactive immature B cells occurs when light chain is rearranged. This tolerance is known as central tolerance [52, 53]. Traditionally, autoreactive B cells that bind to self Ags were thought to be eliminated at the immature B cell stage during central tolerence [54, 55]. However, recent studies indicate that autoreactivity is necessary for B cell development at early stages [56, 57]. The recent hypothesis proposes that a low level of immature B cell expansion will be induced by pre-BCR which is not autoreactive, while pre-B cells with autoreactivity will encounter self-Ag and undergo a proliferative burst. Autoreactivity of newly developed pre-B cells can be removed subsequently by rearranging light chain genes. This process is believed to ensure a broad Ab diversity in the adaptive immune system [58].

1.2.4 T cell dependent B cell activation

1.2.4.1 Germinal centre responses

After maturation in bone marrow and maturation systemically from transitional B cells, naïve B cells expressing surface IgM and IgD circulate in the blood and migrate through secondary lymphoid organs, such as spleen, lymph nodes and tonsil [59]. B cells enter secondary lymphoid tissue via high endothelial venule (HEV) in the T-cell zones, and then migrate towards the B cell follicles [28].

If naïve B cells passing by the border between T-zone and B-zone do not encounter a specific Ag, they would leave the lymphoid organ by efferent lymph vessel, return to the peripheral blood and continue circulating [28].

If naïve B cells encounter Ags and pre-activated CD4+ T cells on the border between the T-zone and B-zone, they would be activated and form or migrate into germinal centre (GC) to proliferate and generate B cells expressing diverse Abs through somatic hypermutation (SHM) and class switch recombination (CSR) [60]. Firstly naïve B cells that have encountered Ags and received cognate T cell help move to the dark zone of GCs and form centroblasts which are rapidly proliferating. Then progeny of those rapidly dividing centroblasts termed centrocytes, express surface Ig and move to the light zone of the GC to test their affinity to Ags [37, 59].

The selection of high affinity B cells in the light zone of GCs has been reported to depend on several signals [61]. Signal through BCR has been demonstrated to

play an important role in selecting high affinity B cells, as well as signals through CD40 and CD40L interaction delivered by T follicular helper (T_{FH}) cells in GCs (details described in section 1.3.3) [61-64]. B cells with high affinity Abs then differentiate into memory B cells [65, 66] or long-lived plasma cells [67, 68] or continue to cycle in the GC and undergo further rounds of mutations and selection [69]. GC B cell differentiation has been reported to be mediated by many signals [61]. Signaling through CD40 has been demonstrated to favour memory B cell development [70, 71]. IL-2, IL-10, CD40L and IL-21 have been found to promote the generation of memory B cells, while IL4 partially inhibits it [61, 72]. IL-10 has also been reported to promote the generation of plasma cells [73], while IL-24 has been reported to inhibit this differentiation [74]. B cells with low affinity for Ags will either go apoptosis or recycle through the GC [69].

1.2.4.2 Somatic hypermutation and class switch recombination

Diversity of Abs in mature B cells after activation with T cell help is generated by somatic hypermutation (SHM) and class switch recombination (CSR) of the Ig genes. SHM alters the Ab affinity to Ag through mutating individual nucleotide in the variable region of both light and heavy chains, while CSR produces functional diversity by switching to different isotypes of antibody harboring the same antigen-binding sites by changing the constant regions of heavy chains.

Activation-induced cyditine deaminase (AID) is a DNA cytidine deaminase that is expressed by activated germinal centre B cells [75]. AID has been reported to be essential for both SHM and CSR [76-78]. Further studies have reported that AID initiates SHM and CSR by deaminating cytosine (C) to uracil (U) [76, 79].

In terms of SHM, it has been reported that following AID deamination, the U-G pair can be transcribed and replicated into a transition mutated T-A pair [76, 79]. Alternatively, the U residue can be removed by uracil-DNA glycosylase (UNG) [80]. The abasic site created by excising of U can either be filled by a C, in which case the AID and UNG attacked point is repaired and no mutation occurs [80]; or be filled randomly by the other three types of nucleotides by translesion polymerases, in which case SHM from C to G or A, or T occurs [28]. The U-G mismatch can also be recognized by MSH2-MSH6 complex instead of UNG [81]. In the presence of MSH2-MSH6, exonuclease I and low fidelity polymerase η has been reported to trigger the mutation of upstream and downstream A-T [28].

In the case of CSR, AID initiates the recombination by deaminating C in switch (S) regions, which are located upstream of each C region [82]. Similar to SHM, the produced U residues are then removed by UNG. Instead of refilling the abasic residue directly, the apurinic/apyrimidinic endonucleases (APE1 and APE2) generate double strand breaks (DSBs) which are essential for CSR [83]. Then donor and acceptor S regions containing double strand breaks align and recombine together [82].

1.2.5 T cell independent B cell activation

Although T cells are required for B cell responses involving GC formation, some microbial constituents, can support B cell responses in the absence of cognate T cell help [28]. According to different stimulating mechanism, thymus-independent (TI) Ags are divided into two classes, TI-1 and TI-2.

It has been reported that high concentration of TI-1 Ags can activate nonspecific B cell responses , which is known as polyclonal activation [84].

Lipopolysaccharide (LPS) a TI-1 Ag in mice that can bind to TLR-4 on B cells [85].

It has been reported that B cell IgA CSR is up-regulated by DCs when stimulated with LPS and native choleva toxin (nCT) during TI-1 response [86].

TI-2 Ags include bacterial capsular polysaccharides that contain highly repetitive structures [87]. It has been reported that TI-2 Ags activate mature B cells through cross-linking many BCRs that are specific for the Ag [88]. Some studies have demonstrated that both recirculating B cells and B cells from GCs can differentiate into cells that can respond to TI-2 Ags [89]. It has been demonstrated that DCs and macrophages can support TI-2 Ag induced B cell activation by producing B cell survival factors, such as BAFF and a proliferation-inducing ligand (APRIL) (details described in section 1.2.6) [90, 91].

1.2.6 B cell survival factors

1.2.6.1 B cell activating factor (BAFF)

B cell activating factor (BAFF), which is also known as B Lymphocyte Stimulator (BLyS) and TNF- and APOL-related leukocyte expressed ligand (TALL-1), is a cytokine that belongs to the tumor necrosis factor (TNF) ligand family [92]. BAFF is encoded by the *TNFSF13B* gene on chromosome 13q32-34 in human [92, 93]. In the human BAFF encoding gene, there are 6 exons: exon 1 encodes the transmembrane domain; exon 2 encodes the furin processing site; and exons 3 to 6 encode the TNF homology domain binding to receptors [94]. In human, BAFF can be either expressed on the cell surface or released as a soluble trimeric ligand [94]. BAFF can be expressed on a range of different cells, including monocytes, activated neutrophils, DCs and T cells [95-97]. There are three receptors for BAFF: transmembrane activator-1 and calcium modulator and cyclophilin ligand-interactor (TACI), B cell maturation antigen (BCMA) and BAFF receptor (BAFF-R) [35].

1.2.6.2 A proliferation-inducing ligand (APRIL)

A proliferation-inducing ligand (APRIL), also known as tumor necrosis factor ligand superfamily member 13 (TNFSF13), is a protein that belongs to the TNF ligand family [98]. APRIL is encoded by the *TNFSF13* gene on chromosome 17 in human [94]. It is a 250aa protein which contains an extracellular domain (encoded by exons 2 to 6), a hydrophobic transmenbrane domain (encoded by

exon 1) and a predicted cytoplasmic domain in human [98]. Similar to BAFF, APRIL can either be expressed on the cell surface or released as soluble trimers [94].

In contrast to BAFF, APRIL can only bind to TACI and BCMA, but not BAFF-R in human [94, 99]. Although BAFF and APRIL can both bind to BCMA, the binding affinities are different [99]. It has been reported that the APRIL: BCMA interaction has higher affinity than BAFF: BCMA interaction [100, 101]. TACI has been demonstrated to interact with BAFF and APRIL with similar affinity [101, 102]. However, the mechanisms of binding of TACI to BAFF and APRIL are different, as TACI can bind to both BAFF trimers and BAFF multimers [103] and APRIL does not form multimers [104]. Recently, some studies have reported that APRIL can also interact with heparan sulphate proteoglycan (HSPG) [105].

1.2.6.3 Function of BAFF and APRIL

BAFF and APRIL have been shown to play an important role in B cell survival and proliferation [35, 94]. The interaction between BAFF and BAFF-R on B cells is essential for B cell survival and differentiation at immature stages [103, 106, 107]. It has been reported that immature transitional B cells require BAFF signaling to develop into mature B cells [108]. APRIL has been shown to play a critical role for later-staged B cells and plasma cells through reaction with its receptors TACI or BCMA [109, 110]. Huard, et al (2008) demonstrated that

APRIL upregulates the expression of anti-apoptotic factors Bcl-2 and Bcl-X_L [111]. It also has been shown that over-expression of Bcl-2 and Bcl-X_L can rescue B cells in BAFF-R deficient mice demonstrating that interaction between BAFF and BAFF-R encourages B cell survival through preventing apoptosis [112, 113]. Moreover, BAFF and APRIL have been reported to induce class switch recombination (CSR) in human without help from T cells [95]. Some studies have reported that BAFF-R and TACI can initiate Ig isotype switching in B cells [114, 115]. The interaction between BAFF and BAFF-R has been reported to support T cell proliferation as well [116, 117].

Increased levels of serum BAFF have been detected in patients with autoimmune diseases [35]. Antibody to BAFF (Belimumab) is a new therapeutic option for treatment of autoimmune diseases [118], implying that BAFF plays important role in B cell survival in autoimmune diseases.

1.3 T cell development

1.3.1 T cell maturation

Naïve T cells develop in the thymus from stem cells that originate in bone marrow. Similar to B cells, T cell maturation progresses alongside rearrangement of the genes encoding the T cell receptor (TCR) in thymus [28]. The development of T cells in thymus is divided into three stages: double negative (DN), double positive (DP) and single positive (SP) [119]. The DN stage can be subdivided into 4 stages according to the expression of CD25 and CD44: DN1 (CD44+CD25-), DN2 (CD44+CD25+), DN3 (CD44-CD25+) and DN4 (CD44-CD25-) [120, 121] .

It has been reported that TCR rearrangement of ɣ, δ or α and β loci occurs at the DN3 stage and after this stage cells successfully rearranged TCR ɣ and δ chains will express ɣδ TCRs and then proceed along the ɣδ lineage pathway [120, 122]. In contrast, cells that successfully rearrange TCR β chain express pre-TCR, which is formed by a rearranged β chain and a surrogate α chain while cells that failed TCR β chain rearrangement die in DN4 stage [119, 120]. This pre-TCRs lead T cells to stop rearranging β chain but further proliferate and express both CD4 and CD8 on their surfaces, which are termed double positive (DP) T cells [28]. TCR α chain rearrangement occurs in CD4+CD8+ T cells [122].

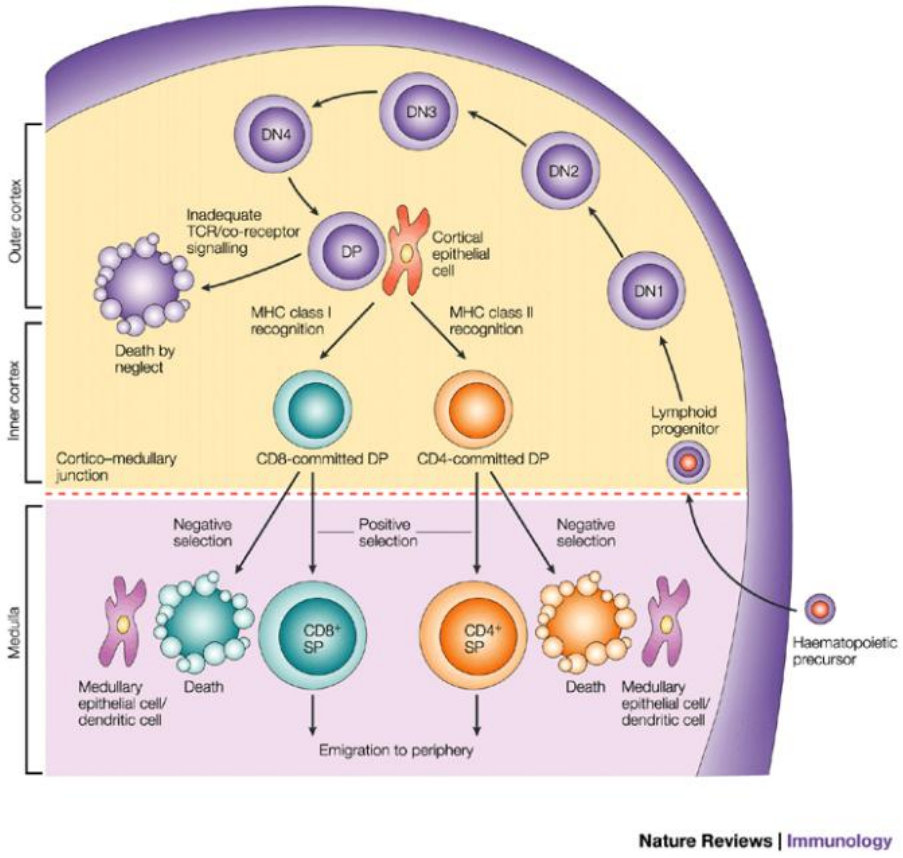

Figure 1.3.1 Overview of T cell development in thymus [123].

When α chain rearrangement is completed, DP T cells expressing TCRs which are low affinity to self major histocompatibility complex (MHC) and peptide complex can go through positive selection (less than 5%) and develop to either CD4 or CD8 SP T cells. The others die [119, 120]. To prevent potential auto-reactivity, T cells with TCR with too high affinity to self MHC: peptide complex will be eliminated through negative selection and die [120]. It has been suggested that negative selection can occur throughout all stages of T cell development, and may not be sequential to the process of positive selection [28]. The overview of T cell development is shown in Figure 1.3.1.

1.3.2 Effector T cell subsets

After differentiation and migration to lymphoid tissue, naïve T cells may be activated by antigen presenting cells that express suitable peptide: MHC complex and then become effector cells. There are many types of effector T cells divided by the different cytokines they produced.

Cytotoxic T cells are CD8+ T cells that recognize MHC-I molecules. It has been reported that CD8+ T cells can release Fas ligand (Fas-L) which can trigger apoptosis in target cells, INF-ɣ and TNF-α [124].

In CD4 T cells, there are several different effector cells, for example T helper 1 (Th1) cells, T helper 2 (Th2), T helper 3 (Th3) cells, T helper 9 (Th9) cells, T helper 17 cells (Th17), T follicular helper (T_{FH}) cells, regulatory T (Treg) cells and T regulatory 1 (Tr1) cells.

Th1 cells activate macrophages. The main cytokine T_H1 cells produce is IFN-ɣ, which can stimulate infected cells and promote inflammation [28].

Th2 cells are involved in immune responses to parasites, and allergy. Th2 cells can support B cell activation by producing cytokines important for B cell proliferation, such as IL-4, IL-5 and CD40-L [28].

Th9 cells have been reported to induce inflammation in animal models [125, 126]. Some studies have reported that Th9 cells are able to produce IL-9, IL-10 and IFN-ɣ [127, 128].

Th17 cells have been reported to promote acute inflammation by producing cytokines to recruit neutrophils to the infected tissues. Cytokines produced by Th17 cells are mainly IL-17 family and IL-6. Th17 cells have been reported to be associated with autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis [129].

T_{FH} cells are T cells found in GCs to provide help for B cell activation and high affinity Ab production. They have been reported to express high levels of chemokine receptor CXCR5 and produce IL-21 and IL-4 [130] (details described in section 1.3.3).

Treg cells have been reported to down-regulate immune response by secreting suppressive cytokines TGF-β and IL-10 [131, 132] (details described in section 1.3.4).

Th3 cells and Tr1 cells have been reported to be subsets of inducible Treg cells [133]. Th3 cells can produce TGF-β in mice [134, 135], while Tr1 cells have been reported to produce IL-10 [133].

1.3.3 Follicular helper T (T_{FH}) cells

1.3.3.1 T_{FH} cell differentiation

T follicular helper (T_{FH}) cells are a CD4+ T cell subset that resides in GCs in B cell follicles of secondary lymphoid tissues [59]. It has been reported that T_{FH} cells can differentiate either from naïve T cells directly once activated or from Th2 cells [136].

When naïve T cells are pre-activated by DCs, they up-regulate B-cell lymphoma 6 (Bcl-6) which is a transcriptional repressor that has been reported to be important for GC formation and to limit differentiation to other CD4+ T cell subsets, such as Th1, Th2, and Th17 cells [137, 138]. Bcl-6 expression has been reported to be essential for T_{FH} cell differentiation and function, although the mechanism is not clear yet [136, 138-140]. It has been reported that Bcl-6 can down-regulate expression of CCR7 and P-selectin glycoprotein ligand-1 (PSGL-1), which guide cells to localize to T cell zones, and up-regulate CXCR5 expression on T_{FH} cells, which allow them to migrate to GCs [140-143]. Interaction between inducible co-stimulator ligand (ICOS-L) expressed on B cells and DCs and ICOS expressed on T_{FH} cells has been reported to support T_{FH} cell differentiation and function by activating phophatidylinositol-3-kinase (PI3K) signaling [144, 145]. In addition, cytokines such as IL-21, IL-6 and IL-27 have been reported to be able to promote T_{FH} differentiation [144, 146, 147].

Some studies have reported that differentiation of T_{FH} cells from activated naïve T cells is independent of other CD4+ T cell subsets [148]. Other studies have demonstrated that T_{FH} cells can be derived from Th2 cells in mice [149]. This differentiation has been shown to be GC dependent which implies that B cells and other GC specific molecules play important roles in T_{FH} cell differentiation from Th2 cells [149].

It has been reported that clones of T_{FH} cells disseminate widely and circulate as a minority subset of T cells in the blood [150]. CD4+ T cells expressing CXCR5 in human peripheral blood have been found to be able to migrate to GCs [151]. Although circulating CD4+ CXCR5+ T cells in peripheral blood may have similar functions to T_{FH} cells from GCs, the precise relationship between these cells is not clear yet.

1.3.3.2 Markers for T_{FH} cells

It has been reported that T_{FH} cells express CXCR5 which is the receptor for CXCL13 produced in follicles of secondary lymphoid tissues [152, 153]. Some studies have reported that T_{FH} cells express ICOS [154]. Other studies have demonstrated that programmed death-1 (PD-1) which is an inhibitory molecule of CTLA-4 family (details described in section 1.7.1) is up-regulated on T_{FH} cells [155]. CD57 expression by T_{FH} cells have been found in GCs of human tonsils, lymph nodes and spleen [130, 156]. However, it has been reported that CD57 is not expressed on all T_{FH} cells in GCs [157]. Therefore, T_{FH} cells are identified as CD4+CXCR5^{hi}ICOS^{hi}PD-1^{hi} [130].

A small subset of circulating T cells has the phenotype and functional properties of T_{FH} cell. A recent study has identified CD4+CXCR5^{hi}ICOS^{hi} or CD4+CXCR5^{hi}PD-1^{hi} circulating T_{FH} cells in peripheral blood from SLE patients by comparing with T_{FH} cells in tonsils [158]. Cells in the blood with phenotype and properties of T_{FH} cells are referred as circulating T_{FH} (cT_{FH}) cells.

1.3.3.3 Function of T_{FH} cells

The direct contact between TCR on T_{FH} cells and peptide: MHC-II complex on B cells, the binding of CD40L and CD40, ICOS and ICOS-L, and cytokines secreted by activated T_{FH} cells, including IL-21 and IL-4, are all essential for activation and proliferation of B cells in germinal centres [130]. IL-21 secreted by T_{FH} cells has

been reported to induce B cell differentiation and to regulate GC responses and Ig production [159-162]. T_{FH} cells have also been reported to produce IL-4 and IFN-ɣ which can induce Ig CSR [130, 163, 164]. Other studies have reported that T_{FH} cells can produce IL-17 which contributes to GC formation in autoimmunity [165]. Interaction between PD-1 expressed on T_{FH} cells and PD-1 ligands expressed on GC B cells has been reported to regulate B cell survival and generation of plasma cells [67, 166].

1.3.4 Regulatory T (Treg) cells

1.3.4.1 Treg cell differentiation

Regulatory T (Treg) cells are a subset of CD4+ T cells found in peripheral blood that can down-regulate immune responses by suppressing cell proliferation and cytokine production [167]. Treg cells can be derived from either thymocytes during early development in thymus (termed natural Treg cells, nTreg) or from T cells in the periphery (termed inducible Treg cells, iTreg) [168].

It has been reported that the differentiation of Treg cells in thymus is mediated by intermediate strength TCR signal, FoxP3 expression, NF-κB signaling and cytokines [168]. Studies of mice have observed Treg cell expansion in thymus when TCR signaling is enhanced [169]. A major subset of Treg cells is defined by the expression of transcriptional factor FoxP3 that regulates the Treg

suppressor function and differentiation in thymus [168]. Some studies have reported that FoxP3 expressing Treg cells will survive while FoxP3 negative Treg cells will be eliminated when receiving the same TCR signal [170]. Increased FoxP3 expression on Treg cells has been found in thymus of mice when the signal strength of NF-κB pathway is enhanced [171]. It has also been demonstrated that deficiency in IL-2, IL-15 and IL-7 results in depletion of FoxP3+ Treg cells in thymus [172, 173].

The gut-associate lymphoid tissues (GALT) and mesenteric lymph nodes have been reported to be the places where inducible Treg cells are generated [168]. In contrast to natural Treg cells, inducible Treg cells have been reported to be generated more efficiently under stimulation of high affinity TCR signaling in the periphery [174]. Transforming growth factor beta (TGF-β) has been reported to promote the differentiation from naïve T cells to inducible Treg cells [175, 176].

1.3.4.2 Markers for Treg cells

When first identified, Treg cells were characterized by high expression of CD25, which is the α-chain of IL-2 receptor, on activated CD4+ T cells [167, 177]. Then transcription factor FoxP3 was identified as a specific marker for Treg cells by further studies as it is only expressed on Treg cells but not other CD4+ T cell subsets [178, 179]. Recently, Miyara *et al* have divided $FoxP3^+$ $CD4^+$ Treg cells into three distinct populations based on expression of CD25 and CD45RA, which

represent different stages of differentiation including: naïve or resting Tregs expressing CD45RA and intermediate levels of FoxP3 and CD25; memory Tregs expressing intermediate levels of FoxP3 and CD25 and lacking expression of CD45RA; and activated Tregs expressing high levels of FoxP3 and CD25 and lacking expression of CD45RA [180].

1.3.4.3 Function of Treg cells

The molecular mechanisms of Treg cells induced immune suppression are not fully understood [168]. It has been reported that Treg cells suppress immune responses through secreting anti-inflammatory cytokines, such as IL-10 and TGF-β [131, 132]. IL-10 produced by Treg cells has been demonstrated to down-regulate immune responses at environmental interfaces [181], and to inhibit the production of IL-12 therefore down-regulate Th1 responses [182]. TGF-β has been reported to inhibit Th1 cell differentiation while promoting Th17 cell differentiation in animal models [183].

In addition, Treg cells have been reported to induce cell apoptosis in T responder cells and antigen presenting cells [184, 185]. Recently, some studies have reported that Treg cells in GCs play an important role in controlling germinal centre responses [186, 187].

It has been reported that CD25 expressed on Treg cells can bind to IL-2 therefore inhibit effector T cell differentiation [188]. Cytotoxic T-lymphocyte antigen 4

(CTLA-4) has also been considered to contribute to Treg suppressive activity as it has been reported that Treg cells in CTLA-4 deficient mice lose their suppressive capacity [189, 190].

1.4 Monocytes

1.4.1 Innate immunity

The innate immune system forms the earliest immunological barriers to infection. In contrast to the adaptive immune system, innate immune responses to pathogens are fast but without long-term immunological memory [191]. When microorganisms invade tissue, they could be recognized immediately by dendritic cells (DCs), macrophages which are continuously matured from monocytes and neutrophils, [28]. Cells in innate immune system recognize microorganisms through pattern recognition receptors (PRRs) expressed on the cell surface. PRRs recognize repetitive patterns termed pathogen-associated molecular patterns (PAMPs) expressed only on microorganisms surface but not on the self cells. Once activated through PRRs, DCs can activate naïve T cells therefore induce an adaptive immune response. Toll-like receptors (TLRs) are a type of PRRs. TLRs expressed by DCs have been shown to be an important link between innate and adaptive immunity. TLR

pathways have been shown to regulate the production of IL-12 and IL-18 which signal naive T-cells to mature into type 1 helper T cells, and to upregulate the expression of costimulatory molecules, such as CTLA-4, PD-1 and PD-Ls (details described in section 1.7), which play essential roles in T-cell activation. Regulated recognition of self and non-self by cells of innate immune system therefore plays an important role in prevention of autoimmune diseases.

1.4.2 Monocytes

Monocytes are part of innate immune system. They develop from precursors in bone marrow, and can further differentiate into macrophages and dendritic cells (DCs) [192]. Monocytes can be divided into subsets by expression of different chemokine receptors and surface markers [193, 194].

In mice, monocytes have been identified as CD115+CD11b+ cells. They can be subdivided into two functional subsets according to the expression of Ly6C, which is a glycosylphosphatidylinositol-anchored molecule [192, 194]. It has been reported that CD115+Ly6C+ monocytes in mice express high levels of CCR2 and low levels of CX3CR1. They have been reported to be recruited to inflammatory tissues and produce pro-inflammatory cytokines, such as TNF-α and IL-1 [194, 195]. Another subset of monocytes in mice has been identified as CD115+Ly6C- cells which express low level of CCR2 while high level of CX3CR1 [192, 194]. These monocytes have been demonstrated to patrol blood vessels, and to be involved in repairing damaged tissues [196, 197].

In humans, monocytes have been characterized by the expression of CD14 and CD16. It has been reported that CD14 expression identifies over 90% of monocytes in humans [194]. CD14+ monocytes can be further divided into three subsets in humans by their expression of CD16: the majority classical monocytes which are $CD14^{hi}$CD16- cells expressing high level of CCR2 while low level of CX3CR1; the intermediate monocytes which are $CD14^{hi}$CD16+ cells expressing low level of CCR2 and high level of CX3CR1; and the non-classical monocytes which are CD14+$CD16^{hi}$ cells expressing low level of CCR2 and high level of CX3CR1 [192, 194]. It has been reported that the classical monocytes resemble murine CD115+Ly6C+ monocytes [194]; the intermediate monocytes produce pro-inflammatory cytokine TNF [198]; while non-classical monocytes patrol blood vessels and play an important role in anti-virus immune responses [199].

1.5 Tissue specific lymphocyte migration

After activation, lymphocytes migrate to different target tissues through peripheral blood [28]. This migration is site specific and guided by adhesion molecules, chemokines selectively produced in different organs and chemokine receptors expressed on lymphocyte surface [200, 201].

Integrins, selectins, Ig superfamily members and vascular addressins are the main classes of adhesion molecules that play important roles in lymphocyte trafficking [202]. Interactions between selectins expressed on lymphocytes and vascular addressins on the endothelium allow lymphocytes to roll along the endothelium of blood vessels. Then lymphocytes adhere to the endothelium by firmer interaction between integrins on lymphocyte surface and adhesion molecules expressed on endothelium. Subsequently lymphocytes migrate into tissue by diapedesis [203].

Integrin molecules α4β7 and α4β1 expressed on lymphocyte surface have been reported to identify two different populations homing to mucosal organs [204, 205]. Lymphocytes expressing high levels of α4β7 are guided to the gastrointestinal tract through binding to endothelial adhesion molecule MadCAM-1 [204]. In addition, mucosal thymus-expressed chemokine (TECK) (also known as CCL25) is expressed predominantly by small bowel epithelium and mucosae-associated epithelial chemokine (MEC) (also known as CCL28) by colonic epithelium. These chemokines attracts cells expressing chemokine

receptors CCR9 and CCR10 to the small bowel and colon respectively. Most of these cells are $\alpha4\beta7^{hi}$ [205-208]. In contrast, most $\alpha4\beta7^{low}$ lymphocytes highly express α4β1 integrin which is a receptor for vascular cell adhesion molecule 1 (VCAM-1), which mediates migration to non-gastrointestinal sites [205]. Chemokine receptor CCR10 can also be expressed on $\alpha4\beta7^{low}$ cells that home to skin, bronchi, nasal and oral mucosa through interaction with cutaneous T-cell-attracting chemokine (CTACK) (also known as CCL27) [205, 209]. In addition, cutaneous lymphocyte antigen (CLA) which is a lymphocyte receptor for E-selectin has been reported to facilitate lymphocyte movement to inflamed skin and oral mucosa [205, 210, 211].

1.6 Important pathways in Autoimmunity

1.6.1 B cells and Autoantibodies

It has been known for a long time that antoantibodies produced by auto-reactive B cells are a consistent feature of autoimmune diseases [212].

The autoantibody response has different targets in different diseases and consequences of autoantibody production vary [28]. For example,

autoantibodies that bind red blood cells can lead to red cell destruction and autoimmune hemolytic anemia [213]. It has been reported that autoantibodies against the thyroid-stimulating hormone receptor stimulate the hormone production constantly in Graves' disease [214, 215]. In other autoimmune diseases, autoantibodies have been reported to cause tissue damage through binding to extracellular self-Ags [216].

The mechanisms driving autoantibody production are not clear yet. It has been suggested that T_{FH} cells helping B cells in GCs would play an important role in regulating autoantibody production [217, 218] (details described in section 1.3.3).

As B cells can be Ag presenting cells as well, they are also considered to have the potential to play a role in activating auto-reactive T cells in autoimmune diseases [28].

1.6.2 Self-reactive T cells

Apart from helping autoreactive B cells to produce autoanibodies, self-reactive effector T cells have also been reported to damage tissues directly in some autoimmune diseases [219]. Specific cytotoxic T cells targeting insulin-producing β cells have been reported in type I diabetes [220]. Auto-reactive CD4+ T cells have been observed in central nervous system

producing inflammatory cytokines therefore promoting lymphocyte infiltrating in patients with multiple sclerosis [28]. Similarly, it has been reported that auto-reactive CD4+ T cells can be activated by DCs and produce pre-inflammatory cytokines and chemokines therefore cause tissue damage in patients with rheumatoid arthritis (RA) [221].

1.6.3 T_{FH} cells in autoimmunity

Germinal centre T_{FH} cells are involved in the progression of autoimmunity in a mouse model of SLE by lowering the threshold for B cell survival in GCs [222]. This lack of stringency in T cell mediated B-cell selection supports the evolution of autoreactive antibody secreting clones of cells. Thus, dysfunctional or excessive numbers of T_{FH} cells in germinal centres have been implicated in the pathogenesis of autoimmune diseases by permitting the survival of autoreactive B cells [222].

ICOS is expressed on T_{FH} cells at high levels. Decreased autoantibody production and organ injury in ICOS deficient mice has been observed which again implies an important role of T_{FH} cells in autoimmunity [218]. Cytokines produced by T_{FH} cells have also been reported to contribute to autoimmune diseases. Studies have reported that IL-21, IFN-ɣ and IL-4 produced by T_{FH} cells play an important role in autoantibody production and tissue injury in mouse models for SLE [217, 223].

There is evidence that T_{FH} cells may be involved in autoimmune responses in humans in a subset of patients with severe SLE, where the proportion of circulating T_{FH} cells, which were defined as CXCR5+CD4+ T cells expressing high levels of ICOS or PD-1, was significantly higher than that in healthy controls [158].

1.6.4 Immune suppression by Treg cells and autoimmunity

As described above, the function of Treg cells is to suppress immune responses. Treg cells have been thought to be differentiated from self-reactive T cells in thymus that then develop strong suppressive function to suppress self reactivity [28]. Therefore a defect in Treg function would be likely to play an important role in the pathogenesis of autoimmune diseases. Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) which is a disease first described in 1982 that often includes autoimmune symptoms such as diabetes. It has been reported to be due to a defect in Treg cells caused by FoxP3 mutations [224, 225].

Defect in Treg cell function can be caused either by a change in Treg cell frequency or a deficiency in suppressive capacity or a functional failure of cells that respond to Treg cells. There are many examples of Treg deficiency in autoimmune diseases. For example, studies have observed normal numbers of

Treg cells with deficiency in suppressive capacity in patients with multiple sclerosis [226]. It has been reported the frequency of Treg cells in peripheral blood is associated with disease activity in patients with rheumatoid arthritis (RA) [227], while other studies have reported defective Treg cell suppressive function in patients with RA [228].

1.7 Programmed death-1 and its ligands

1.7.1 Co-stimulatory molecules

In addition to Ag-specific signals, co-stimulatory molecules are required to fully activate B cells and T cells [28]. In absence of second signal from co-stimulatory molecules, T cells activated through TCR-MHC complex interaction will be turned into anergy. It has been reported that co-stimulatory molecules CD28, CTLA-4, ICOS and PD-1 and their ligands play important roles in immune responses. The expression, function and interaction of co-stimulatory molecules and their ligands is shown in figure 1.7.1 [229]. Co-stimulatory molecules can regulate T cell responses through different mechanisms. For example, expression of CTLA-4 on T cells has been reported to enhance T cell movement and therefore stop T cells interacting with APCs for too long during

activation in GC [230, 231]. It has also been suggested by other studies that CTLA-4 can inhibit CD28 co-stimulatory signal by trans-endocytosing the ligands CD80 and CD86 which are shared with CTLA-4 (Figure 1.7.1) [232]. In addition, CTLA-4 deficiency has been demonstrated to impair Treg cell suppressive function in mouse model [190].

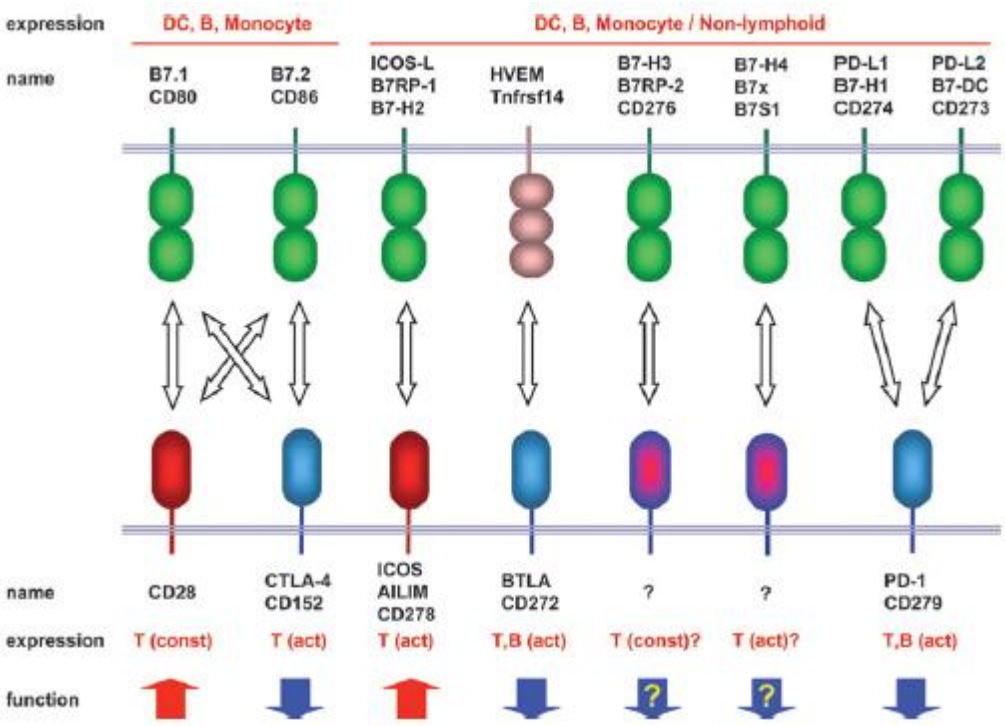

Figure 1.7.1: Expression, interaction and function of co-stimulatory molecules [229].

1.7.2 Programmed death-1 (PD-1)

1.7.2.1 Structure of PD-1

Programmed death-1 (PD-1) is a member of the immunoglobulin superfamily that associated with programmed cell death [233]. It is encoded by the *pdcd1*

gene on chromosome 1 in mice and chromosome 2 (2q37) in human [234, 235]. *Pdcd1* consists of 5 exons: exon 1 encodes signal sequence; exon 2 encodes the IgV domain; exon 3 encodes the stalk and the transmembrane domain; exon 4 and 5 encode the cytoplasmic domain [234]. The mature form of the PD-1 protein is 268 amino acids (aa). It contains an N-terminal IgV-like domain, followed by an approximately 20 amino acid stalk, then a transmembrane domain and a cytoplasmic domain [236]. The cytoplasmic domain of PD-1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) which has been shown to be essential for PD-1 function in T cells and B cells [236, 237].

1.7.2.2 Expression of PD-1

The expression of PD-1 has been reported to be hardly observed on resting cells [236]. Some studies have observed PD-1 expression on immature CD4-CD8- T cells, and the expression of PD-1 can be induced on activated CD4+CD8+ T cells in mice [238]. It has been reported that PD-1 expression can be induced on activated T cells and B cells through TCR or BCR signaling [234, 239]. In contrast to activated T cells, Treg cells in both mice and human have been reported to retain PD-1 intracellularly [240]. It has been reported that the level of mRNA encoding PD-1 is not correlated with the level of protein expressed on cell surface [239]. In addition, PD-1 has been reported to be inducibly expressed on activated antigen presenting cells (APC) in human [241].

In other studies, PD-1 has been identified as a marker for T follicular help (T_{FH}) cells in germinal centres in human [242, 243]. Recently, the high expression of PD-1 together with chemokine receptor CXCR5 has been reported to be able to identify circulating T_{FH} cells in human peripheral blood [158]. In addition, the increased proportion of PD-1 high expressing circulating T_{FH} cells in peripheral blood has been reported to be associated with certain subset of SLE patients. This implies that the expression of PD-1 on T_{FH} cells could play a role in autoimmune diseases [158].

1.7.2.3 Signalling through PD-1

Ligation of PD-1 with its ligand PD-L1 or PD-L2 has been reported to inhibit T cell proliferation, cytokine production and impair T cell survival through TCR signal [235]. The extent of PD-1 mediated inhibition has been reported to be dependent on the strength of the TCR signal; the bigger inhibition is induced by the weaker TCR signal [234, 236]. It also has been reported that the PD-1 pathway can inhibit cytokine production more efficiently than cell proliferation, although the mechanism is unclear [234, 236].

In contrast to the cytotoxic T-lymphocyte antigen 4 (CTLA-4) pathway, engagement of PD-1 has been demonstrated to inhibit T cell responses through blocking the induction of phophatidylinositol-3-kinase (PI3K) and the downstream activation of Akt [244]. Further studies illustrated that tyrosine phosphatase 1 (SHP-1) and tyrosine phosphatase 2 (SHP-2) can bind to the ITSM

motif in the cytoplasmic domain of PD-1, and play an essential role in the PD-1 inhibitory pathway [237, 245]. Binding of SHP-1 and SHP-2 with the ITSM motif of PD-1 could result in increased expression of phosphatase and tensin homolog (PTEN), which can efficiently block the PI3K pathway [236]. As the CTLA-4 pathway blocks the Akt signal but does not affect PI3K activity, the inhibition induced by PD-1 ligation has been reported to be greater extent than CTLA-4 pathway [244]. Some studies have reported the inhibition mediated by PD-1 can be overridden by co-stimulating with CD28 or IL-2 [246, 247].

1.7.2.4 Function of PD-1

PD-1 deficiency in animal studies has been shown to affect lymphocyte development and functions. One study reported that PD-1 deficiency results in increased double negative T cells in thymus and spleen in mice [248]. Another study on PD-1 deficient mice demonstrated that PD-1 negatively regulates the transition of double negative T cells to double positive T cells in thymus [249]. Other studies on PD-1 deficient mice illustrated that lack of PD-1 results in impaired peripheral CD8+ T cell tolerance and self-reactive CD8+ T cell responses in lymph nodes [250, 251]. Although the percentage of Treg cells in CD4+ T cells remained at normal level in mice deficient in PD-1, Treg cells from these mice was found to have reduced suppressor function [252]. Other studies of PD-1 deficient mice demonstrated that B cell responses are negatively regulated by PD-1, which can also influence B cell differentiation [253], while

more IgA with reduced bacteria-binding capacity was induced in the gut of PD-1 deficient mice [254].

1.7.2.5 PD-1 polymorphisms in autoimmune diseases

An association between PD-1 and human autoimmune disease has been identified by genomic studies. A susceptibility locus for SLE was identified on chromosome 2q37 by a genomic screen in 2000 [255, 256]. Subsequently, the *pdcd1* gene encoding PD-1 was located within the region 2q37.3 and was found to associate with susceptibility in SLE [257]. PD1.3A allele has been reported to associate with renal manifestations in patients with SLE by a Swedish group [258]. Another study has found the PD1.3A allele is significantly associated with SLE susceptibility in Caucasian patients [259]. Others have found more PD1.3A alleles in patients with SLE in Greece [260], but less in SLE patients in Spain [261]. Another study reported that the PD1.3A allele is significantly associated with SLE in Latin Americans but not in European and African SLE patients [262]. In addition to SLE, polymorphisms in *pdcd1* have also been reported to associate with rheumatoid arthritis (RA) in patients from Iran and Taiwan [263, 264], type 1 diabetes in patients from Denmark [265], and ankylosing spondylitis from patients in China [266].

1.7.3 Programmed death-1 ligand 1 (PD-L1)

1.7.3.1 Structure of PD-L1

Programmed death 1 ligand 1 (PD-L1) is one of the ligands of PD-1 which is also known as cluster of differentiation 274 (CD274) or B7 homolog 1 (B7-H1) [246]. It is a type I transmembrane protein of the B7 family which is encoded by the *CD274* gene on chromosome 19 in mice and chromosome 9 in human [234, 267]. The *CD274* gene consists of 7 exons: exon 1 is a non-coding gene; exon 2 encodes a signal sequence; exon 3 encodes the IgV domain; exon 4 encodes the IgC-like domain; exon 5 encodes the transmembrane; exon 6 and 7 encode the cytoplasmic domain [234]. PD-L1 is a 290 aa protein [234]. It contains a IgV-like domain and a IgC-like domain, followed by a transmembrane domain and a short cytoplasmic domain [236]. The cytoplasmic domain of PD-L1 is approximately 30 aa and found to be conserved in many species [234, 236]. However, the function of the intracellular part of PD-L1 is still unknown [246, 267]. One study has reported mutated PD-L1 encoding mRNA lacking IgV-like domain in human [268].

1.7.3.2 Expression of PD-L1

PD-L1 has been reported to be widely expressed on many different cell types, including both hematopoietic and non-hemopoietic cells [236]. Some studies have observed PD-L1 expression on T cells, B cells, DCs, macrophages, stem cells

and bone marrow derived mast cells in mice [269]. In humans, PD-L1 has been found to be expressed in heart, lung, placenta and skeletal muscle [234, 267]. Studies on cell lines have demonstrated that the expression of PD-L1 is dependent on myeloid differentiation primary response gene 88 (MyD88), TNF receptor associated factor 6 (TRAF6) and MAPK/ERK kinase (MEK) pathways [270]. Another study has reported that blocking phosphatase and tensin homolog (PTEN) could result in increased expression of PD-L1 [271]. It has been reported that PD-1 expression is dependent on interferon (IFN) regulatory factor-1 binding site [272], and can be up-regulated by IFNα and IFNβ stimulation [273, 274]. It has been reported that interleukin-2 (IL-2), IL-7 and IL-15 can increase the PD-L1 expression on human T cells, while IL-21 can increase the expression of PD-L1 on human B cells [275]. In contrast, IL-10 has been reported to induce PD-L1 expression on monocytes [234].

Apart from hematopoietic cells, PD-L1 can also be constitutively expressed on many non-hematopoietic cells, such as vascular endothelial cells, pancreatic islet cells, and neurons [234, 236, 267].

1.7.3.3 Signaling through PD-L1

Ligation of PD-1 with its ligand PD-L1 or PD-L2 has been reported to inhibit T cell proliferation, cytokine production and impair T cell survival through TCR signal [235]. However, apart from affecting immune responses through TCR or BCR signaling, PD-L1 can also deliver inhibitory signals to the cells expressing

PD-L1 [234]. One study on mice has demonstrated that the over-expression of PD-L1 on DCs does not alter T cell response, while ligation of PD-L1 expressed on DCs with soluble PD-1 can inhibit the DC activation and increase IL-10 production suggests there is a reverse signaling by PD-L1 to DCs [276].

PD-1 is not the only receptor for PD-L1. B7-1 co-stimulatory molecule has been reported to be another receptor for PD-L1 through their IgV-like domains [277]. The B7-1: PD-L1 interface has been reported to partly overlap with the PD-1: PD-L1 interface [277, 278]. It has been demonstrated that interaction between B7-1 and PD-L1 inhibits T cell activation and cytokine production [277]. Since PD-L1 and B7-1 are expressed on many different cell types, people hypothesis the bidirectional interactions between PD-L1 and B7-1 may have potential influence in immune responses [234].

1.7.3.4 Function of PD-L1

PD-L1 deficiency in animal studies has been shown to be associated with autoimmune diseases. Studies on mice have shown that PD-L1 deficiency can cause expansion of autoreactive T cells with diabetes [279, 280]. Another study on PD-L1 deficient mice demonstrated that PD-L1 expressed on T cells, antigen presenting cells (APCs) and host tissues negatively regulates the T cell cytokine production [281]. Despite inhibiting T cell responses, PD-L1 has also been demonstrated to potentially influence T cell differentiation. Studies on mouse models have reported that PD-1/PD-L1 interaction down-regulated the

expansion of T_{FH} cells but up-regulated the development of Treg cells [166, 282]. Moreover, PD-L1 is thought to associate with Treg function as one animal study indicating that PD-L1 deficient Treg cells were defective in inhibiting Th1 responses in mice [283]. Recently, Treg cells were shown to suppress autoreactive B cells through a PD-1/PD-L dependent pathway [284].

Polymorphisms of PD-L1 encoding gene *CD274* have been reported to be associated with Graves' disease in Asian and Caucasian cohorts [285, 286], and with ankylosing spondylitis in Chinese patients [266, 287]. In contrast, polymorphisms of *CD274* have been reported to not be associated with RA in Taiwan [288] or SLE in many different cohorts [289, 290].

1.7.4 Programmed death-1 ligand 2 (PD-L2)

1.7.4.1 Structure of PD-L2

Programmed death 1 ligand 2 (PD-L2) is one of the ligands of PD-1 which also known as cluster of differentiation 273 (CD273) or B7-DC [291, 292]. It is a member of the B7 family which is encoded by the *pdcd1lg2* gene on chromosome 19 in mice and chromosome 9 in human [234]. *Pdcd1lg2* gene consists of 7 exons in human: exon 1 is a non-coding gene; exon 2 encodes signal sequence; exon 3 encodes the IgV domain; exon 4 encodes the IgC-like domain; exon 5 encodes the short stalk, transmembrane and the beginning of the cytoplasmic tail;

exon 6 and 7 encode an approximately 30 aa cytoplasmic domain [234]. The approximately 30 aa cytoplasmic domain is conserved across many mammals. The function of the intracellular tail of PD-L2 is unknown [234, 236]. Studies have reported splice variants in PD-L2 encoding mRNA in human [291, 293]. One mutation results in the loss of the IgV-like domain of the PD-L2 protein would result in failure of binding to PD-1 [278, 291]. Another two splice variants have been reported to affect the IgC-like domain with or without affecting the transmembrane domain in PD-L2 [268]. However, these mutations would be expected not to affect the binding capacity to PD-1 as the IgV-like domain has been reported to be the domain that has most binding activity to PD-1 [278].

1.7.4.2 Expression of PD-L2

In contrast to the wide expression of PD-L1 on many different cell types, the expression of PD-L2 is more restricted [234, 236]. Expression of PD-L2 by activated cells from peripheral blood in human has been described. One study indicated that PD-L2 is inducibly expressed by activated CD4+ and CD8+ T cells from human blood [294]. PD-L2 expression by peripheral blood monocytes was reported to be up-regulated in multiple sclerosis patients treated with IFN-β [295].

The expression of PD-L2 in tissues has also been described in autoimmune mouse models and human studies. In human, PD-L2 has been found to be

expressed by macrophages and DCs in livers from patients with autoimmune liver diseases [296]. Other studies have shown that PD-L2 is expressed by renal tubular epithelial cells in the kidneys from patients with lupus nephritis [297]. Constitutive expression of PD-L2 has been detected in un-stimulated human umbilical vein and brain endothelial cells, while the expression was found to be up-regulated under inflammatory conditions [298, 299]. PD-L2 expression has also been observed on inflammatory cells in rheumatoid synovium from patients with rheumatoid arthritis [300].

There is no agreement on the expression of PD-L2 in tissues of mice. Some studies indicated that PD-L2 was not expressed by activated lymphocytes in the spleen, bone marrow and thymus of mice [301]. Another study observed PD-L2 expression on DCs in thymus from naive mice and on inflammatory infiltrating cells in brains from EAE mice [302]. This was supported by an observation of PD-L2 expression on DCs from mesenteric lymph nodes in mice [303].

1.7.4.3 Signaling through PD-L2

Like the PD-1/PD-L1 pathway, the interaction between PD-L2 and PD-1 has been shown to inhibit T cell proliferation and cytokine production in mouse models and human studies [291, 304, 305]. Studies have reported the PD-1/PD-L2 pathway down-regulates CD4+ as well as CD8+ T cell responses in mice [247]. This was further confirmed by PD-L2 blockade in EAE mice, which results in T cell expansion and accelerated and more severe disease [306]. Similar to PD-L1,

PD-L2 has also been reported to regulate the generation of inducible Treg cells in mice. One study has demonstrated that PD-L2 deficient DCs induce fewer Treg cells in mesenteric lymph nodes in mouse models [303]. In addition, PD-L2 has also been reported to be involved in regulating autoreactive B cell responses, as the expression of PD-L1 and PD-L2 on Treg cells were both required for inhibiting autoreactive B cell proliferation in mice [284].

Similar to PD-L1, PD-L2 has been reported to deliver inhibitory signal to the cells expressing PD-L2 [234]. One study on mice has demonstrated that ligation of PD-Ls expressed by DCs with soluble PD-1 can inhibit the DC activation and increase IL-10 production suggesting that there is a reverse signaling by PD-Ls to DCs [276].

1.7.4.4 Function of PD-L2

PD-L2 deficiency has been shown to result in experimental autoimmune encephalomyelitis in a mouse model, with a less severe disease and inflammatory cytokines produced in lymph nodes than the PD-L1 deficient mice [307].

Polymorphisms of PD-L2 encoding gene *pdcd1lg2* have not been thoroughly studied. It has been reported that polymorphisms of *pdcd1lg2* are not associated with the occurrence of RA in Taiwan [288]. Another study has reported association between *pdcd1lg2* polymorphisms and ankylosing

spondylitis in patients from Taiwan [287]. It has been reported that the polymorphisms of *pdcd1lg2* are associated with SLE in patients from Taiwan [289], but not SLE patients from Sweden, Mexico or Argentina [290].

1.7.5 The PD-1/PD-L pathways

The PD-1/PD-L pathways have been demonstrated to affect cell maturation and proliferation in animal models. PD-1/PD-L1 interaction has been found to be required to induce T cell anergy before T cells migrate from lymphoid organs to peripheral blood in mice [308]. Further study of mouse models has indicated that blockade of the PD-1/PD-L1 pathway leads to less T cell movement but longer interaction between T cells and DCs in lymph nodes [309]. Moreover, interaction between PD-1 and its two ligands has been shown to inhibit CD4+ and CD8+ T cell responses in mice [247]. Autoreactive B cells in mice have been demonstrated to be suppressed by PD-1 ligation through PD-L1 and PD-L2 [284], while disruption of PD-1/PD-L pathways in mice have been reported to result in fewer plasma cells [67]. DC maturation in mice has been shown to be inhibited through PD-1/PD-L pathways as well [276].

The effect of PD-1/PD-L pathways on T cell proliferation in human has also been reported. PD-1/PD-L pathways have been reported to down regulate both primary and secondary T cell responses in peripheral blood from healthy donors [305]. The interaction between PD-1 and PD-L2 has been shown to inhibit T cell proliferation as well as cytokine production in human blood [304]. Further

study has indicated that PD-1/PD-L pathways negatively regulate T cell proliferation in human through affecting multiple regulators of the cell cycle [310].

1.8 VISTA

V-domain Ig suppressor of T cell activation (VISTA) is a newly identified Ig superfamily ligand which down regulates T cell responses [311]. VISTA is a 309 aa type 1 transmembrane protein which contains a IgV-like extracellulcar domain, a stalk domain, a transmembrane domain and a cytoplasmic domain. The extracellular IgV-like domain of VISTA is homologous to PD-L1. However, VISTA does not bind to PD-1 and the receptor for VISTA is unknown. The expression of VISTA has been observed on many hematopoietic cells, especially T cells and APCs, and some organs such as lung, brain and kidney. It has been shown that VISTA expressed on APCs suppresses T cell proliferation and cytokine production in mouse models. Anti-VISTA treatment has been reported to exacerbate the development of autoimmune disease in animal models [311].

1.9 Immunology of GPA

1.9.1 Hypothesis of autoantibody production in GPA

Friederich Wegener first observed that patients with this disorder almost universally had upper airway disease especially affecting the facial sinuses [312, 313]. As upper airway inflammation is so often the first sign of GPA, infections are suspected to trigger the disease. It has been suggested that *Staphylococcus aureus* infection may be associated with the pathogenesis of GPA and persistent *S. aureus* nasal carriage is associated with a risk of disease relapse [314, 315]. Anti-neutrophil cytoplasmic antibody (ANCA) has been reported to be closely associated with GPA through reacting with proteinase 3 (PR3) [13, 316]. It has been shown that rising levels of ANCA in GPA patients' blood can predict the relapse of the disease [13, 316]. Abundant PR3 expression has been detected in lung biopsies of GPA patients by immunohistochemistry, where most PR3 positive cells were macrophages [317]. The increased percentage of neutrophils expressing membrane PR3 has been shown to associate with relapse of GPA [318]. In addition, Reumaux et al, 1995, demonstrated that membrane PR3 on resting neutrophils was two to three times less than neutrophils activated by inflammatory factors [319]. Thus, a current hypothesis for the mechanism of vessel damage in PR3-ANCA associated vasculitis is that monocytes activated by infection release proinflammatory factors, such as IL-1 and TNF-α, which up-regulate the expression of adhesion molecules on

endothelium and stimulate neutrophils to express membrane PR3. As a consequence, activated neutrophils adhere to endothelium of vessel walls. Meanwhile, in the presence of PR3 released by activated macrophages, B cells activated by pathogen, with T cell help, produce ANCA. Subsequently, neutrophils adhered to endothelium are stimulated by ANCA through PR3-ANCA reaction, leading to degranulation and release of reactive oxygen species which results in vascular damage [314, 315]. However, how ANCA is produced by B cells and how PR3-ANCA reaction induces vasculitis are still not clear.

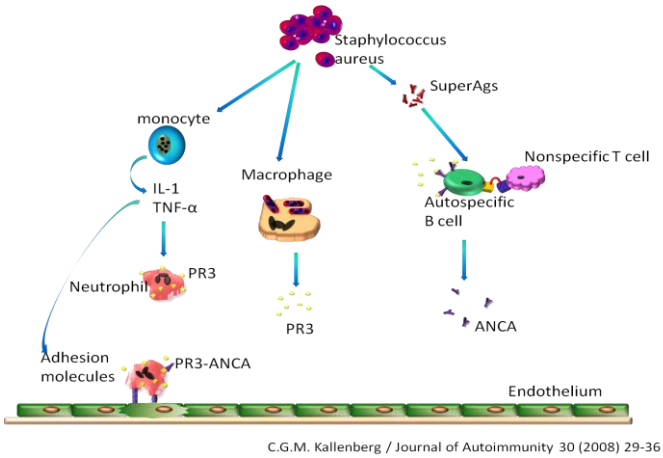

Figure 1.9.1 Hypothesis of infection triggering PR3-ANCA associated vasculitis [314]

Consistent with this hypothesis, innate and adaptive immune systems have both been reported to contribute to the chronic inflammation seen in GPA. Dendritic cells (DCs) have been reported to help T cell responses and potentially contribute to granuloma formation in GPA [320]. Another study has demonstrated that both macrophages and neutrophils may play a role in the

inflammation in GPA [321]. Other studies have observed expansion of T cells and B cells in granulomatous lesions from GPA patients [322, 323].

1.9.2 Current studies of B cells in GPA

The types of lymphocyte infiltrating in granuomatous lesions from GPA patients are various. Formation of follicle-like B cell infiltrates have been observed in granulomatous lesions from GPA patients [324, 325]. Others have reported the presence of germinal centre-like structures with follicular dendritic cells in granulomatous lesions from GPA patients [326]. Gene analysis of the Ig heavy chains of B cells isolated from granulomatous lesions from GPA patients has shown mutations [323-325]. This implies that B cells in the lesions are generated by germinal centre responses. Ig gene analysis also identified clonal expansion of B cells in lesions from GPA patients [323]. Apart from ANCA against PR3 [327], B cells in lesions from GPA patients have been found to produce other Abs specific to a proinflammatory transmembrane protein [328].

ANCA has been demonstrated to play an important role in the pathogenesis of GPA [329]. Rituximab which is a monoclonal antibody against CD20 has been shown to efficiently deplete B cells which could develop into ANCA-producing plasma cells [330] and has therefore been considered as a treatment in GPA patients [331]. Two large randomized controlled trials have clearly demonstrated the clinical benefits of rituximab in GPA patients with active and

relapsing disease and this agent has been approved by the Food and Drug Administration [24, 332]. Interestingly, B cells have been observed in liver lesions from a GPA patient following rituximab treatment while circulating B cells were absent from peripheral blood [333]. This implies B cells in the lesions of GPA patients might not be a component of circulating B cells in peripheral blood.

1.9.3 Current studies of Treg cells in GPA

Regulatory T cells (Treg) are critical regulators of autoimmune diseases through suppressing the proliferation and cytokine production of effector T cells [334, 335]. The failure of regulatory T cells (Treg) to suppress inflammation, either through defects of Treg or the cells that respond to them, may facilitate the development of autoimmune pathology. Some studies have shown Treg frequencies to be reduced in GPA, and other studies have detected defects in the suppressive function of Treg in GPA. Both of these sets of observations implicate Treg in the disease process [336-338]. Interestingly, Treg frequencies have been shown to increase in the blood of patients with SLE following rituximab treatment [339]. This could be a secondary consequence of clinical remission, or if Treg are actually associated with the disease process and are not purely bystanders, this data might imply that Treg are in some way B cell dependent.

1.9.4 Current studies of PD-1, its ligands and VISTA in GPA

The expression of PD-1 and its ligands PD-L1 and PD-L2 by different cell types from GPA patients had not been reported when the studies in this thesis were started. Polymorphisms in the pdcd1 (PD-1 encoding gene), CD274 (PD-L1 encoding gene) and pdcd1lg2 (PD-L2 encoding gene) genes have not been thoroughly studied in GPA. One study has reported that polymorphisms in pdcd1 were not associated with GPA [340]. No disease association with polymorphisms in genes encoding PD-L1 or PD-L2 has been reported in GPA.

As a newly identified Ig superfamily ligand in mouse, the expression of VISTA in GPA patients has not been studied [311].

1.10 Systemic lupus erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a chronic multi-systemic autoimmune disease with wide range of many different symptoms [341]. SLE can affect all ethnicities with a high female to male ratio (approximately 9:1) [342], and higher prevalence in Asia and Africa [343, 344].

The clinical features of SLE include photosenstive skin rashes, arthritis, nephritis, cardiac and pulmonary disease and central nervous system (CNS) disorders, *et al* [35]. Serologically, SLE is characterized by high autoantibody titers and abnormal B cell activation in peripheral blood [345]. It has been reported that autoantibody titers in peripheral blood can be detected many years before the clinical onset of SLE [346], and reach a peak prior to the onset of illness [347].

The pathogenesis of SLE is not fully understood [348]. It is widely agreed that genetic and environmental factors together can trigger SLE [35, 349]. Polymorphisms of many different genes (including genes encoding human leukocyte antigen (HLA), Fc receptor, complement components and PD-1) have been reported to associate with SLE [257, 349]. There are many theories relating to the pathogenesis of SLE. For example B cells, B cell survival factors, cytokines, complements, interferon related pathways and failure in clearance of apoptotic debris have all been implicated in the pathogenesis of SLE [35, 350-352].

SLE and GPA are both systemic autoimmune diseases that can induce nephritis which is a life-threaten symptom. The mechanisms of inducing nephritis in SLE and GPA are different: membrane attack complex of complement system mediated in SLE but vasculitis induced in GPA. Therefore active SLE patients were selected as a disease control for GPA study in this thesis.

1.11 Aims of studies in this thesis

The aims of studies in this thesis were to investigate the immunology in GPA and to increase understanding of this disease.

As the immunological features of the mucosal microenvironment in GPA have not been studied extensively, the aim of chapter 3 was to investigate the immunological features of biopsies from GPA patients. It was hypothesised that B cell clones identified in the mucosa from GPA patients might also circulate in the blood. To investigate this hypothesis, junctional sequences of Igs that are unique to each B cell and its clonal progeny were compared.

As described in section 1.9.2, rituximab has been reported to be a beneficial therapy for GPA patients. However, the effects of B cell depletion therapy on different T cell subsets in general immune system is still unclear. Therefore the aim of chapter 4 was to investigate any difference in frequencies of circulating T_{FH} cells and Treg cells from GPA patients on conventional therapies, GPA patients following rituximab treatment, healthy and disease controls.

As introduced in section 1.7, PD-1 and its ligands are immunoglobulin superfamily members that could be expressed on many different cell types. It has been reported that polymorphisms in genes encoding PD-1 and its ligands are associated with various autoimmune diseases. PD-1/PD-Ls pathways may also affect the generation of different T cell subsets. Therefore, the aim of

chapter 5 was to identify any differences in the expression of PD-1, PD-L1, PD-L2, and VISTA in PBMC from GPA patients, healthy and disease controls.

Chapter 2

Materials and Methods

2.1 Ethics approval

This study was approved by the National Research Ethics Service (Approval No. 10/H0715/3) and by the Guys and St Thomas' Hospital Ethics Committee. All patients donated blood and agreed to allow access to biopsy and tissue samples that were surplus to clinical investigation after fully informed written consent.

2.2 Patients

2.2.1 Patients involved in peripheral blood study

GPA patients attending the systemic vasculitis clinic in the Louise Coote Lupus Unit at St Thomas' Hospital were invited to participate in this study following written informed consent. SLE patients attending lupus clinic in the Louise Coote Lupus Unit at St Thomas' Hospital were invited to participate as disease controls in this study following written informed consent. GPA patients and SLE patients fulfilled the American College of Rheumatology classification criteria [11]. Data on disease activity and damage using the BVAS and the VDI was collected at the time of venesection for GPA patients. Data on British Isles Lupus Assessment Group (BILAG) was collected at the time of venesection for SLE patients. Information on organ involvement and treatment was also collected.

GPA patients who underwent B cell depletion with rituximab did so based on clinical need. These patients had all failed standard therapies with

corticosteroids and immunosuppressive agents and still had active or relapsing disease. All patients gave written informed consent prior to receiving rituximab. Protocol is to discontinue immunosuppressive agents one week prior to rituximab but oral corticosteroids are continued. Patients received rituximab 1g at day 1 and day 15 and were followed with clinical parameters, BVAS, CD19 counts and serum immunoglobulin levels. Patients also received iv methylprednisolone 100mgs and iv chlorpheniramine 10mgs prior to each infusion to minimize the risk of a hypersensitivity reaction.

Approximately age and gender matched healthy control subjects from hospital and laboratory staffs were recruited and gave blood following written informed consent.

The details of clinical information of patients involved in different studies are listed in tables in each result chapter.

2.2.2 Patients involved in biopsy study

Biopsies taken for diagnosis from GPA patients were studied following written consent. The group of patients with orofacial granulomatosis (OFG) were considered to be an important control group because this is a chronic granulomatous mucosal lesion, but not associated with acute inflammation or autoimmunity. The group of patients with sarcoidosis was considered to be a

control group as granuloma is a feature of the disease. Written consents were obtained as appropriate.

2.3 Tissues

2.3.1 Blood samples

Peripheral blood samples were collected in universal tubes containing spray-coated K_2EDTA (BD Vacutainer). Peripheral blood mononuclear cells (PBMC) were isolated with Ficoll-Paque from fresh blood samples. General information of GPA patients and healthy and disease controls involved in each chapter is listed in table 2.3.1. Details of clinical information of GPA patients and SLE controls are listed in tables in each chapter.

Table 2.3.1 General information of patients and controls studied in each chapter

Chapter			Age (years)		
		No.	average	range	M:F
3	GPA	16	53	25 to 73	1:1
	HC	11	45	22 to 68	5:6
4	GPA	27	51	25 to 83	14:13
	BCD GPA	9	58	42 to 73	2:1
	SLE	8	41.5	23 to 65	0:8
	HC	10	45	22 to 68	1:1
5	GPA	10	49	25 to 67	6:4
	BCD GPA	7	58	42 to 73	5:2
	SLE	9	44	22 to 66	0:9
	HC	10	52	34 to 69	1:1

2.3.2 Paraffin biopsies

Paraffin blocks of inflamed mucosal biopsies from 8 patients with clinically confirmed GPA, were selected to include a range of characteristics from mild lymphocytic infiltrates considered within normal histological limits through to marked inflammation (Detailed location of the tissue sampled and the diagnoses are included in Table 2.3.2). Specimens were graded according to the inflammatory infiltrate present to produce a semi-quantitative score (Nil, Mild and Marked). This was undertaken blind by two authors (PP & EO).

Table 2.3.2 Diagnoses and general information of biopsies

Site	Histological Diagnosis	Gender (M:F)	Age	Inflammatory infiltrate			Numbers
			Mean (Range)	Nil	Mild	Marked	
Lip	17x OFG	12:5	27 (10-68)	0/13	12/17	5/17	17
Gingiva	5x OFG	1:4	19 (7-72)	1/5	2/5	2/5	7
	2x GPA (ANCA no data)	1:1	49,73	0/2	0/2	2/2	
Buccal	6x OFG	3:3	37 (9-68)	1/6	3/6	2/6	6
Palate	1x OFG	0:1	9	1/1	0/1	0/1	1
Mediastinal lymph node	1x Sarcoid granuloma	0:1	No data	N/A	N/A	N/A	1
Nasal	1x GPA (ANCA -)	0:1	22	0/1	0/1	1/1	1
Submandibular gland	1x GPA (ANCA -)	1:0	66	0/1	0/1	1/1	1
Bronchial	2x GPA (ANCA+)	1:1*	47, 39*	0/2	0/2	2/2	2

2.4 Solutions

TBS: 6.05g Trisma base (Sigma), 80g sodium chloride and 38ml 1M HCL were dissolved in 10 litres dH_2O and mixed well to make a TBS stock. Concentrated HCL was added a drop at a time until the solution was pH 7.6.

1M Tris: 121.1g Trisma base (Sigma) was dissolved in 800ml dH_2O and mixed well. Concentrated HCL was added a drop at a time to adjust pH value to 9.

10x TBE: 108g of trizma base (Sigma), 55g boric acid and 8.3g EDTA (Sigma) was dissolved in 1L dH_2O by mixing vigorously.

Ethidium Bromide stock (0.5 µl/ml): 10mg ethidium bromide was dissolved in l ml dH_2O and 10µl of this was diluted in 200µl of 1x TBE to produce a concentration of 0.5µl/ml.

SOC medium: 2g of Tryptone, 0.5g Yeast extract, 1ml 1M NaCl and 0.25 ml 1M KCl were added to 97ml dH_2O and autoclaved at 120°C for 20 minutes. The SOC medium was left to cool to room temperature before adding 1ml of filter-sterilized 2M Mg^{2+}.

LB Agar: 1g Tryptone, 0.5g Yeast extract, 0.5 NaCl and 1.5g Bacto agar was dissolved in 100ml dH_2O and autoclaved at 120°C for 20 minutes. The Agar was allowed to cool for 10 minutes before adding 200µl 0.5µl/ml Ampicillin (Sigma), 200µl 0.5µl/ml XGAL (Promega) and 0.5ml 0.1M IPTG. Mix well and pour 20ml into each plate while agar is still warm. To prepare the plate sending out for

sequencing, only 200µl 0.5µl/ml Ampicillin was added into the Agar after cooling down.

RPMI-1640 medium: 1640 medium (Gibco) containing 1% L-glutamine was supplemented with 1% penicillin (Sigma) and 10% heat-inactivated (56°C for 30 minutes) fetal calf serum (FCS) (seraLab).

10x PBS: 80g NaCl, 2g KCl, 14.4g Na_2HPO_4 and 2.4g KH_2PO_4 (all from Sigma) were dissolved in 800ml distilled water. pH value was then adjusted to 7.4 with concentrated HCl. Distilled water was added to make up final volume 1 liter.

FACS buffer: 1ml filtered FCS and 200µl EDTA (Sigma) were added into autoclaved PBS (1:10 diluted from 10x PBS) and mixed.

X-VIVO 15 medium: X-VIVO 15 medium (Lonza) was supplemented with 5% heat-inactivated human AB serum (PAA Labs Ltd), 100µl/ml penicillin/streptomycin (Invitrogen) and 100µg/ml fungizone (Invitrogen)

2.5 Immunohistochemistry staining (IHC)

All reagents were from DAKO unless stated. Paraffin embedded tissue sections (5µm) were baked at 60°C for 30min. Then sections were rehydrated with Xylene for 2x10 minutes and then IMS for 2x10 minutes, followed by distilled water for 10 minutes. Sections were then submitted to antigen retrieval by

heating retrieval solution (DAKO) at 95°C for 40 minutes and then cooled down on bench for 30min and then washed with TBS unless stated. After retrieval, sections were incubated with Dual Endogenous block or Peroxidase block for 10 minutes and then washed with TBS. Sections were then incubated with appropriate primary Ab for 1 hour at room temperature unless stated and then washed with TBS. Sections were then incubated with secondary Abs for 30 minutes, and then washed with TBS. Choice of secondary Ab was depend on different primary Abs: a) Rabbit and mouse Abs were developed with HRP envision; b) Goat Ab (PD-1) was developed with a rabbit anti-goat secondary Ab (dilution 1:100).

For single staining, sections were then developed with DAB+ (diluted 1:100 with DAB substrate buffer) and then washed with TBS, followed by counter staining, and then rehydrating and mounting steps described below.

For double staining, sections were incubated with Double staining block for 10 minutes, and then washed with TBS. Sections were subsequently incubated with appropriate primary Abs for 1 hour at room temperature and then washed with TBS unless stated. Then sections were incubated with Rabbit-mouse Link for 30 minutes followed by Polymer/AP for 30 minutes, and then washed with TBS. Sections were then developed with red chromogen from DAKO or Vector labs, and then washed with distilled water.

After developed with DAB+ or red chromogen, sections were counter stained with haematoxylin for 5 seconds, and then washed with running water. Sections were subsequently re-hydrated with IMS for 2x10 minutes, followed by Xylene for 2x10 minutes, and then mounted with Depex Mounting medium.

Concentrations of each Ab used for IHC experiments are shown in table 2.5.1.

Table 2.5.1 Detailed information of IHC Abs

	Company	Dilution	Retrieval	Incubation
APRIL	Alexis biochemicals	1:500	DAKO S1700	1hr at RT
BAFF	Millipore	1:120	DAKO S1700	overnight at RT
BAFF-R	Alexis biochemicals	1:20	DAKO S1700	1hr at RT
CD138	DAKO	1:30	DAKO S1700	1hr at RT
CD20	DAKO	1:200	DAKO S1700	1hr at RT
CD3	DAKO	1:25	DAKO S1701	1hr at RT
CLA	Biolegend UK	1:50	DAKO S1700	1hr at RT
E-selectin	R&D systems	1:100	DAKO S1700	1hr at RT
FoxP3	abcam	1:100	DAKO S1700	overnight at 4°C
IgA	DAKO	1:100	DAKO S1700	1hr at RT
IgD	DAKO	1:500	DAKO S1700	1hr at RT
IgE	DAKO	1:1000	Tris PH=9	1hr at RT
IgG	DAKO	1:250	DAKO S1700	1hr at RT
IgM	DAKO	1:150	Tris PH=9	1hr at RT
Ki67	DAKO	1:10	DAKO S1700	1hr at RT
Neutrophil Elastase	DAKO	1:100	NA	1hr at RT
PD1	R&D systems	1:10	DAKO S1700	2hrs in RT
PR3	abcam	1:10	NA	overnight at 4°C

2.6 DNA and RNA extraction

DNA samples for IgH gene study in chapter 3 were isolated from paraffin coated biopsy sections using DNeasy Blood & Tissue Kit (Qiagen). Tissues were digested by incubating with proteinase k at 56°C for 3 hours.

RNA samples for IgH gene study in chapter 3 and PD-1/PD-Ls study in chapter 5 were isolated from blood B cells, different T cell subsets and monocytes sorted according to different subsets using miRNeasy Mini Kit (Qiagen). To prevent contamination for RNA isolation, nuclease-free water (Qiagen) was aliquot and all instruments including pipettes, tips and racks, and nuclease-free water was disinfected under UV light for 20 minutes before each experiment.

2.7 Reverse transcription

RNA samples were reverse transcribed to cDNA using Oligo dT primer (Promega) and M-MLV Reverse Transcriptase kit (Promega). To prevent contamination, nuclease-free water (Qiagen) was aliquot and disinfected under UV light for 20 minutes as well as all instruments including pipettes, tips and racks before each experiment.

To reverse transcribe RNA into cDNA, 7µl of RNA, 3µl of dH_2O and 1µl of primer were mixed and incubated at 70°C for 10 minutes. Tubes were then moved to iced water and left on ice for 10 minutes, and then spun down once for 5mins.

Then 4.0µl of M-MLV Buffer, 0.5µl of dNTPs (Promega), 0.5µl of RNase inhibitor (Promega), 1.0µl of M-MLV Reverse Transcriptase (Promega) and 3.0µl of dH_2O were added to make a final volume 20µl followed by incubation at 42°C for 1 hour. Products were extended at 70°C for another 15 minutes and then held at 15°C.

2.8 Amplification of Ig gene rearrangements

2.8.1 General information

The reverse transcribed cDNA and isolated DNA was amplified by semi-nested PCR. Ig heavy chain gene was amplified from V regions (VH1-6) to C regions (Cα, Cγ, Cµ and Cε). Leader and FR1 primers were performed in semi-nested PCR. DNA isolated from paraffin block was amplified from FR3 to J regions. JH external and VLJH internal primers were applied in semi-nested PCR. To search for clonal relationships between cells in biopsies and different circulating blood subsets, clone specific primers designed from IgH sequence isolated from biopsy were applied to all blood subsets and biopsies from the same patient using the FR3-J PCR program.

In each experiment, 3µl $MgCl_2$, 0.5µl 20mm dNTPs, 10µl 5xBuffer, 0.5µl of each 20mM primer, 3µl cDNA, and 31.5µl dH_2O were added to each tube to make the total volume 50µl.

2.8.2 PCR Programs

- **IgH-C:**
    1) 1st round: 95°C for 10 minutes then hold at 54°C, 2µl Taq DNA polymerase (Promega) was added to the tube after holding, followed by 30 cycles of 94°C for 40 seconds, 65°C for 45 seconds and 72°C for 2 minutes, and then extended at 72°C for 10 minutes.
    2) 2nd round: 95°C for 10 minutes then hold at 54°C, 2µl Taq DNA polymerase (Promega) was added to the tube after holding, followed by 30 cycles of 94°C for 40 seconds, 55°C for 45 seconds and 72°C for 2 minutes, and then extended at 72°C for 10 minutes.
- **IgH-J:**
    1) 1st round: 95°C for 10 minutes then hold at 54°C, 2µl Taq DNA polymerase (Promega) was added to the tube after holding, followed by 7 cycles of 94°C for 60 seconds, 64°C for 2 minutes and 72°C for 45 seconds, and then 39 cycles of 94°C for 30 seconds, 62°C for 30 seconds and 72°C for 90 seconds, then extended at 72°C for 10 minutes.
    2) 2nd round: 95°C for 10 minutes then hold at 54°C, 2µl Taq DNA polymerase (Promega) was added to the tube after holding, followed by 7 cycles of 94°C for 60 seconds, 66°C for 2 minutes and 72°C for 45 seconds, and then 35 cycles of 94°C for 30 seconds,

54°C for 30 seconds and 72°C for 90 seconds, then extended at 72°C for 10 minutes.

- **Clone specific (FR3-J):**

  Samples were heated at 95°C for 10 minutes then hold at 54°C. 2µl 1:20 diluted Taq DNA polymerase (Promega) was then added to the tube, followed by 35 cycles of 95°C for 60 seconds, 60°C for 60 seconds and 72°C for 60 seconds, and then extended at 72°C for 10 minutes.

- **Clone screening:**

  Samples were heated at 95°C for 5 minutes and then followed by 30 cycles of 95°C for 30 seconds for denaturation, 45°C for 30 seconds for annealing and 72°C for 90 seconds for elongation and then extended at 72°C for 5 minutes.

2.8.3 Primers used for PCR

Table 2.8.3 Sequences of PCR primers used for PCR

Cα	GGAAGAAGCCCTGGACCAGGC
Cγ	CACCGTCACCGGTTCGG
Cμ	CAGGAGACGAGGGGGAA
Cε1	TGTCCCGTTGAGGGAGCCTGT
Cε2	GGGTCGACAGTCACGGAGGTGGCATT
VH1-Ldr	CCATGGACTGGACCTGG
VH2-Ldr	ATGGACATACTTTGTTCCAC
VH3-Ldr	CCATGGAGTTTGGGCTGAGC
VH4-Ldr	ATGAAACACCTGTGGTTCTT
VH5-Ldr	ATGGGGTCAACCGCCATCCT
VH6-Ldr	ATGTCTGTCTCCTTCCTCAT
VH1-FR1	GGTGCAGCTGGTRCAGTCTGGGCTG
VH2-FR1	CAGRTCACCTTGARGGAGTCTGGTCC
VH3-FR1	GAGGTKCAGCTGGTGGAGTCTGGGGG
VH4-FR1	GGTGCAGCTGCAGGAGTSGGGCSCAGG
VH5-FR1	AGCTGGTGCAGTCTGGAGCAGAGG
VH6-FR1	TACAGCTGCAGCAGTCAGGTCCAGGAC
JH(external)	ACCTGAGGAGACGGTGACCAGGGT
VLJH(internal)	GTGACCAGGGTACCTTGGCCCCAG
VH-FR3	ACACGGCTGTGTATTACTGT

2.9 Gel electrophoresis

2.9.1 Agarose gel electrophoresis

PCR products of immunoglobulin heavy chain V to C (size range 300bp to 500bp) were detected using agarose gel electrophoresis. Agarose gel was prepared with 6g 3:1 agarose and 200ml TBE buffer. 8 μl of PCR product was mixed with 2μl of Gel loading dye (New England Biolabs), and then loaded into gel. 8μl of

phiX74 DNA markers (Promega) was applied to identify the size of the products. Voltage was set to 127V and applied for 60 minutes. Gels were stained in 0.5μg/ml ethidium bromide solution for 30 minutes, and then exposed to UV light. The ladder of the DNA marker and the PCR products can be observed under UV light.

2.9.2 10% Polyacrylamide mini-gel

10% Polyacrylamide mini-gel was used for low molecular weight DNA samples (size smaller than 100bp). 13ml of dH_2O, 2ml of 10x TBE, 5ml of acrylogel 5, 200μl of 10% Ammonium Persulphate (freshly made) and 20μl of TEMED were added by order, mixed and then pour out quickly to make up new gels. 6 μl of PCR product was mixed with 2μl of Gel loading dye (New England Biolabs), and then loaded into gel. 5μl of Low Molecular weight marker (Biolabs) was applied to identify the size of the products. Voltage was set to 125V and applied for 60 minutes. Gels were stained in 0.5μg/ml ethidium bromide solution for 30 minutes, and then exposed to UV light. The ladder of the DNA marker and the PCR products can be observed under UV light.

2.10 RT-PCR

2.10.1 General information

Quantitative PCR's were designed by Applied Biosciences using their Taqman Gene Expression Assay. Five sets of assays were designed for reactions covering the PD-1, PD-L1, PD-L2, VISTA and GAPDH gene segments in human respectively. Assays applied for sorted CD19+ B cells, CD4+ T cells, CD8+ T cells, CD14+ monocytes and whole PBMC from GPA patients and disease and healthy controls.

2.10.2 Protocol

In each experiment, 5.0μl TaqMan Universal PCR Master Mix, 0.5μl 20× Assay Mix and 4.5μl CDNA diluted to 1/5 or 1/10 in RNase-free water was added to each well to make the total volume 10μl. For each cDNA sample, there were three replicates. Cycling as follow: denature (hold) at 95°C for 10 minutes, and then 40 cycles of 95°C for 15 seconds for denature, 60°C for 1 minute for annealing and extention.

2.10.3 Extraction and calculation of quantitative PCR data

1) Reliability of replicates

The number of cycles taken for each of 3 replicates of each reaction to cross the threshold was read. Occasionally data was variable within the 3 replicates. To deal with these data, two rules were applied in the study: first, if two values in

the replicates have less than 0.3 cycles between them, then remove the third value. Second, if there is a difference greater than 1 full cycle of quantitative PCR between replicates, then leave out this experiment because it is inaccurate.

2) Calculation for each value

As the data of quantitative PCR shows the amplified cycles of each sample, it was calculated to the relative amount of cDNA products. To relate the data of each RT-PCR assay, results of GAPDH was chosen to be the standard, and other results were related to it.

In each individual, after the raw data of GAPDH was chosen to be the standard, every other data (PD-1, PD-L1, PD-L2 and VISTA) was calculated in the equation $Y=2^{(X-A)}$ (X=each raw data, A= cycle number of GAPDH). In this way, the standard (GAPDH) was transferred to 1, and other data was relative to it. However, the raw data indicated the numbers of PCR amplified cycles, which meant that the bigger the data was indicating the smaller amount of original RNA. Thus, all data then was applied in the equation of $Y=1/(X*100)$ (X= each data from the first step calculation) to make sure the correct value relationship between each quantitative PCR segment. Then data was applied to the figures and tables.

2.11 Cloning

2.11.1 Gel extraction & DNA purification

PCR products with a cluster of different sizes of bands were separated by size on gel. The gel with the right size of the band was extracted applying the QIAquick Gel Extraction Kit (Qiagen). Other PCR products were purified using MinElute PCR Purification Kit (Qiagen).

2.11.2 Ligation

Ligation reaction was performed in 10µl for each reaction. Reagents were all from Promega. 3µl of PCR product, 1µl of T4 vector, 1µl of T4 ligase and 5µl of ligase buffer were added to eppendorf tube, mixed and then incubated at room temperature for 2 hours.

2.11.3 Transfection

JM109 component cells (Promega) were thawed on ice, then 25µl of cells and 2.5µl of ligation product were transferred to a new eppendorf tube, mixed and then incubated on ice for 20mins. Samples were subsequently heat-shocked at 42°C for 45s, and then immediately moved back to ice for 2mins. Then samples were incubated with 950µl of SOC medium at 37°C with shaking at 150rpm for 2hrs. Samples were then centrifuged at 200rpm at room temperature for 10mins and then supernatants were taken off. Cells were re-suspended in

100µl SOC medium and spread on Agar plates, incubated at 37°C upside down overnight.

2.11.4 Picking up colonies

Bacteria with a vector containing a DNA insert grew as white colonies and the bacteria with a vector without a DNA insert grew as blue colonies. The white colonies were picked up at random with a pipette tip and transferred to a gridded plate. The gridded plate was then incubated at 37°C upside down overnight. The tip was washed off in 15µl dH_2O.

2.11.5 Screening

2.11.5.1 Protocol

To release the vector and denature the DNAse, dH_2O containing washed off bacteria from the tips was boiled for 10mins and then centrifuged at 400g for 10mins. 5µl of bacterial supernatant was taken off and amplified by PCR using M13 forward and M13 reverse primers. PCR products were then run on a 3% agarose gel to screen for inserts of the correct size.

2.11.5.2 PCR reaction

Each PCR reaction contained 9.46µl nuclease free dH_2O, 4µl 5x flexi buffer (Promega), 1.2µl $MgCL_2$ (Promega), 0.2µl 20mm dNTPs (Promega), 0.02µl of each 100mm M13 primers, 0.1µl Taq polymerase (Promega). PCR program has

been described in section 2.8.2. Sequences of M13 primers are shown in table 2.11.5.

Table 2.11.5 Sequences of M13 primers used for clone screening

M13 Fwd	5'-GTAAAACGACGGCCAGT-3'
M13 Rev	5'-GGAAACAGCTATGACCATG-3'

2.11.6 DNA sequencing

Colonies with appropriately size DNA inserts were tapped with clean tips from the gridded plate to sequencing plate and then sent for sequencing by Beckman Coulter sequencing services.

2.12 Flow cytometry

2.12.1 Cell surface staining

Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood or buffy coats and then washed with 2ml RPMI-1640 medium. Samples were then spun down at 1200rpm or 400g at 4°C for 10 minutes, and then supernatants were taken off. Samples were re-suspended in 2ml 1640 medium and cells were counted under microscope with trypon blue. Cells ($1x10^6$ to $10x10^6$ depending on different experiments) were transferred into FACS tubes (Bioscience) and then washed with FACS buffer. Samples were then spun down at 1200rpm or 400g at 4°C for 10 minutes, supernatants were then taken off. Cells were re-suspended with certain amount of FASC buffer (depending on

volumes of Abs added in, to make up a final volume 200ul) and incubated with fluorochrome-conjugated mAbs on ice for 20 minutes. After incubation, samples were washed once with 2ml FACS buffer, and then spun down at 1200rpm at 4°C for 10 minutes, and then supernatants were taken off. Cells were re-suspended with 500µl FACS buffer (samples too sticky or containing too many cells will be transferred to a new tube through a strainer) and then analyzed with the BD FACSAria I cell sorter or BD FACSCanto. All samples were gated and analyzed in a blinded manner.

2.12.2 Intracellular staining

In some experiments PBMC were also examined for expression of FoxP3 using the FoxP3 Fix/Perm buffer set (eBioscience) according to the manufacturers' instructions.

After step stained with surface Abs, cells were re-suspended in 1ml FoxP3 fix/perm buffer (1:4 diluted) and vortex, and then incubated at room temperature in dark for 45 minutes. Cells were then washed with 2ml Perm buffer (1:10 diluted) and centrifuged at 400g for 5 minutes. Supernatants were then taken off. Samples were subsequently re-suspended in 200µl Perm buffer (1:10 diluted), and incubated with FoxP3 Ab at room temperature in dark for 30 minutes. After incubation, samples were washed with 2ml Perm buffer (1:10 diluted) and then centrifuged at 400g for 5 minutes. Supernatants were taken off after centrifuge. Samples were then washed once with 2ml FACS buffer and

then centrifuged at 400g for 5 minutes. Supernatants were then discarded. Then cells were re-suspended with 500µl FACS buffer and then analyzed with the BD FACSAria I cell sorter or BD FACSCanto.

2.12.3 Ab panels

Table 2.12.3.1 Abs for study of CLA expressing B cells

Ab	Fluorochrome	Company	Volume (µl/200µl)
CD19	PE	BD Biosciences	20
$\alpha_4\beta_7$	PE-Cy7	BD Biosciences	20
CLA	FITC	Biolegend	30
CCR10	APC	R&D system	14

Table 2.12.3.2 Abs for study of circulating T_{FH} cells

Ab	Fluorochrome	Company	Volume (µl/200µl)
CD3	APC-H7	BD Biosciences	5
CD4	Pacific Blue	Biolegend	2.5
CXCR5	PE	R&D system	20
PD-1	APC-C7	BD Biosciences	10

Table 2.12.3.3 Abs for study of Treg cells

Ab	Fluorochrome	Company	Volume (µl/200µl)
CD4	Pacific Blue	Biolegend	2.5
CD14	APC-Cy7	Biolegend	3
CD25 (2A3)	PE	BD Biosciences	5
CD25 (M-A251)	PE	BD Biosciences	10
CD127	PerCp-Cy5.5	eBioscience	5
PD-1	APC	BD Biosciences	10
CD45RA	AF488	Biolegend	5

Table 2.12.3.4 Abs for study of FoxP3 staining for Treg cells

Ab	Fluorochrome	Company	Volume (µl/200µl)
CD4	Pacific Blue	Biolegend	2.5
CD14	APC-H7	Biolegend	3
CD25 (2A3)	PE	BD Biosciences	5
CD25 (M-A251)	PE	BD Biosciences	10
CD127	PerCp-Cy5.5	eBioscience	5
FoxP3	AF647	BD Biosciences	20
CD45RA	AF488	Biolegend	5

2.13 Treg cell suppressive function study

For isolation of cell populations for functional studies, PBMC were stained as described above and regulatory (CD4+CD127loCD25hi) and effector (CD4+CD127+CD25-/lo) T cell populations isolated using a BD FACS-Aria II flow cytometer and FACS Diva Software. *In vitro* co-culture suppression assays were performed by culturing effector T-cell populations ($2.5x10^3$/well) in the presence or absence of autologous Tregs at different ratios: 0:1, 1:1, 1:2 or 0:1. Cells were activated by the addition of Dynabeads® Human T-Activator anti-CD3/anti-CD28 beads (Invitrogen, Paisley, UK) at a bead:conventional-cell ratio of 1:1, 1:2 or 1:4. All assays were conducted in triplicate. After 5 days of culture, proliferation was assessed by the addition of 0.5 µCi/well 3[H]-Thymidine (Perkin Elmer, Waltham, MA, USA) for the final 18 hours of co-culture in X-VIVO 15 media. The percentage of suppression was calculated using the following formula: %suppression = 100-(counts per minute (cpm) in

the presence of Tregs ÷ cpm in the absence of Tregs x 100). All analyses were performed in a paired manner with one GPA and one control individual analyzed on a single day.

2.14 Statistical methods

Data sets are expressed as average and range. All data has been tested for normality. Some of the data studied in this thesis were not normally distributed, therefore nonparametric statistics including Mann Whitney test were used to analyze data in this thesis. Statistical analysis was carried out using GraphPad Prism4.

Chapter 3

Study of B cells in lesions and peripheral blood from GPA patients

3.1 Introduction

GPA is a rare autoimmune disease characterized by granulomatous vasculitis of the upper and lower respiratory tracts and glomerulonephritis [2, 4]. As a systemic inflammatory disease, many organs may be affected in GPA such as lungs, bronchi, oral or nasal mucosa, eyes, kidneys, nerves, brain, joints and skin [3]. However, upper respiratory tract involvement especially the affected facial sinuses was observed to be the most characteristic and common feature of GPA [3, 312, 313]. Moreover, the nasal mucosa has been reported to often be the site of initial disease activity [315, 353]. Therefore it has been proposed that the disease is initiated in the upper airways [312, 313]. It has been suggested that *Staphylococcus aureus* infection may be associated with the pathogenesis of GPA and persistent *S. aureus* nasal carriage is associated with a risk of disease relapse [314, 315].

Anti-neutrophil cytoplasmic antibodies (ANCA) against proteinase 3 (PR3) have been reported to be highly sensitive and specific in GPA patients [13, 316] . Previous studies have found PR3 expressing granulocytes and macrophages in lung tissue from GPA patients [317], and an increased proportion of neutrophils expressing high levels of PR3 in peripheral blood from GPA patients compared to healthy controls [314, 354]. Moreover, an elevated level of PR3 expressed on the surface of neutrophils in peripheral blood was observed to be significantly associated with an increased disease relapse in GPA patients [318]. The

chronological sequence of upper airway symptoms followed by systemic manifestations is consistent with the notion that GPA may evolve as a consequence of upper airway inflammation and that other systemic, potentially life-threatening symptoms are a direct consequence of the activity of pathogenic autoantibodies.

The immunological features of the mucosal microenvironment in GPA have not been studied extensively. Classical features of the disease include a mixed infiltration of acute and chronic inflammatory cells, vasculitis, granulomas and small numbers of giant cells [3, 4, 355, 356]. B cells in normal oro-nasal mucosa and bronchial mucosa have been identified in organized lymphoid structures termed nasal-associated lymphoid tissue (NALT) or bronchus-associated lymphoid tissue (BALT) respectively [357]. In addition isolated activated B cells can be identified beneath the stratified/ pseudo stratified epithelium in oral mucosa that are increased in frequency and are associated with plasma cell differentiation in local inflammatory responses [358]. B cells and antibody producing plasma cell infiltrates were observed to be present in the mucosa of endonasal lesions from GPA patients [325]. Analysis of the immunoglobulin heavy chain genes from granulomatous lesions has found mutations in their immunoglobulin variable regions [323, 325], which is a feature of germinal centre origin [59]. However, conventional germinal centre stuctures have not been reported as a consistent feature of the inflamed airway mucosa in GPA in

previous studies. One study has reported diffusely infiltrating lymphocytes, T cell and B cell infiltrates and germinal centre-like structures with DCs in granulomatous lesions of GPA [326].

Previous studies of animal models and humans have demonstrated that class switch recombination can be induced by BAFF and APRIL in a T cell independent way [95, 114, 359]. As a B cell survival factor, BAFF has been shown to prevent cell apoptosis and support B cell survival in mouse models [106, 108, 112]. In addition, over-expression of BAFF has been shown to lead to autoimmune symptoms in mice [35, 360]. Previous studies have observed higher plasma levels of BAFF in PR3-ANCA vasculitis patients [361] and an increased level of serum BAFF in peripheral blood from GPA patients [362] and activate MPO-ANCA vasculitis patients [363], although the correlation between BAFF and Ab status is not coincided.

It has been reported that lymphocytes could migrate to different target organs by expressing a panel of chemokine receptors and adhesion molecules [205]. Certain subsets of T cells have been reported to be recruited to skin and contribute to the local inflammation in inflammatory skin diseases [210]. Another study has observed that a certain subset of T cells disappeared from peripheral blood but was found in lesions in activate GPA [314]. This raises the question of whether certain subsets of lymphocytes are recruited to inflammatory sites from peripheral blood in GPA patients.

Therefore the immunological features of the mucosal microenvironment in GPA were studied by immunohistochemistry; and B cell clonality in biopsies and peripheral blood were investigated by Immunoglobulin heavy chain (IGH) gene study in this chapter.

Aims of this chapter:

The aim of this chapter is to investigate the immunological features of biopsies from GPA patients, and to investigate any possibility that B cell clones identified in the mucosa might also circulate in the blood by comparison of junctional sequences that are unique to each B cell and its clonal progeny.

3.2 Results

3.2.1 Study of B cells and autoantibody production in biopsies from GPA patients

B cells and local environment in biopsies from 8 patients with GPA were studied through IHC staining. B cells were found to be consistently present in mucosal biopsies from all GPA patients studied. One nasal biopsy from a patient with GPA contained abundant organised mucosa-associated lymphoid tissue (MALT), but the affected mucosa in the remaining cases had no evidence of germinal centre formation. B cells in most cases were found not to be components of any organized lymphoid structure but were scattered submucosally (Figure 3.2.1 A).

Plasma cells were also observed in biopsies from GPA patients. The plasma cell and B cell infiltrates tended to occupy adjacent areas of mucosa. Plasma cells were often observed in the infiltrate that was predominantly B cells, but B cells were rare in the plasma cell dominated zones (Figure 3.2.1 B).

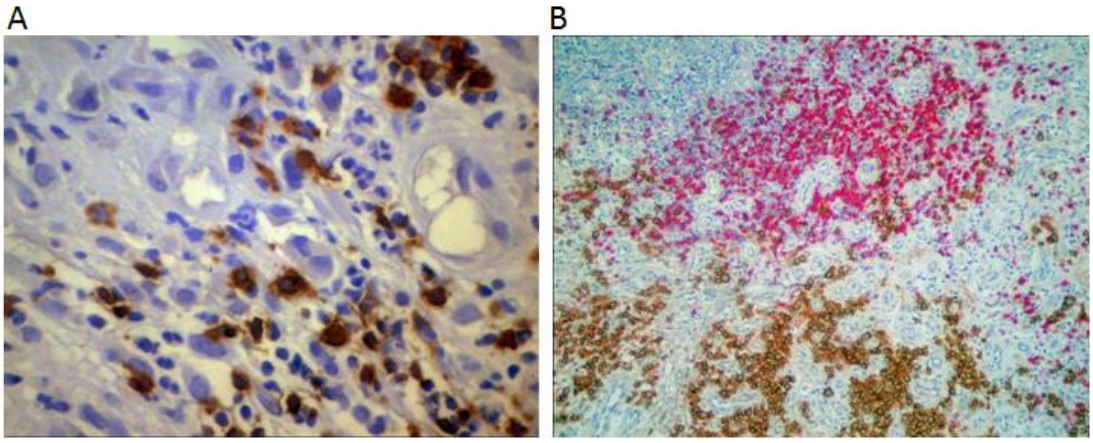

Figure 3.2.1: Examples of B cells in paraffin sections of GPA mucosa studied by immunohistochemistry. **A.** Single staining with CD20 (brown) identified B cells in biopsies from GPA patients, original magnification: x400. **B.** Double staining with CD20 (pink) and CD138 (brown) identified B cells and plasma cells occupying adjacent areas in GPA, original magnification: x200. Plasma cells were present in the B cell dominated areas while B cells did not tend to infiltrate plasma cell dominated areas.

The B cells in biopsies from GPA patients were large cells with extensive dendritic processes that extended and made contact with many adjacent cell types (Figure 3.2.1) (observation was made blindly by two researchers). Approximately 5% of the B cells were found expressing the Ki67 nuclear proliferation antigen (Figure 3.2.2).

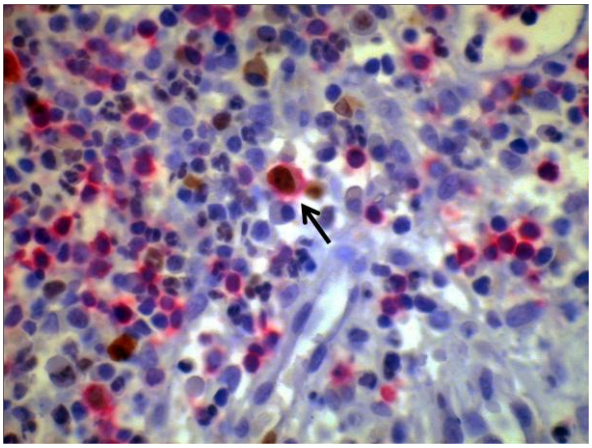

Figure 3.2.2: Example of proliferating B cells in paraffin sections of GPA mucosa studied by immunohistochemistry. Double staining of cell division antigen Ki67 (brown nucleus) and surface CD20 (in pink) identified B cells that were proliferating in the biopsies (arrow pointing out an example), original magnification: x400.

Neutrophils which are a target of autoantibody ANCA in GPA were observed in all biopsies from GPA patients and always located adjacent to B cells (Figure 3.2.3 A). Double staining of PR3 which is the target of cANCA that is expressed on neutrophils, and CD20 confirmed that PR3+ neutrophils present in biopsies from GPA patients were located close to B cells (Figure 3.2.3 B). The B cell infiltrate in GPA was generally intimately mixed with acute inflammatory cells that included cells expressing the GPA associated autoantigen PR3 and neutrophil elastase. CD20+ B cells were observed in germinal centres while CD3+ T cells were found in T cell zones in tonsil sections (Figure 3.2.3 D). In contrast, although germinal centre structures were not observed as a feature of GPA biopsy, CD3+ T cells and CD20+ B cells were observed in adjacent areas in biopsies from GPA patients (Figure 3.2.3 C).

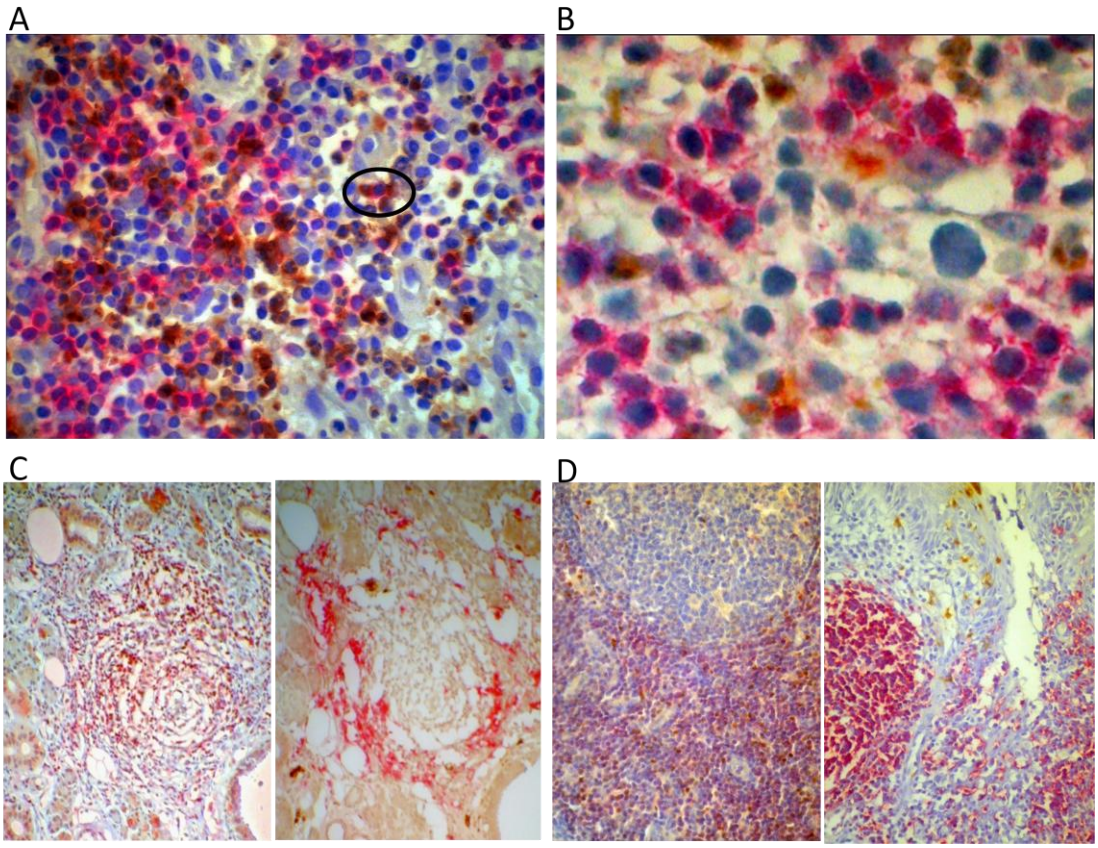

Figure 3.2.3: Examples of B cells and neutrophils in paraffin sections of GPA mucosa studied by immunohistochemistry. A. Double staining of CD20 (pink) and neutrophil elastase (brown) identified that activated B cells were adjacent to neutrophils (an example is circled), the target of autoantibody ANCA, in GPA biopsies, original magnification: x400. **B.** Double staining of PR3 (brown), the main target of ANCA in GPA, and CD20 (pink) original magnification: x600. **C.** Left: Double staining of FoxP3 (brown) and CD3 (pink) on GPA biopsy; Right: Double staining of neutrophil elastase (brown) and CD20 (pink) on GPA biopsy. **D.** Left: Double staining of FoxP3 (brown) and CD3 (pink) on tonsil section; Right: Double staining of neutrophil elastase (brown) and CD20 (pink) on tonsil section.

To study the isotypes of Abs produced in lesions from GPA patients, biopsies were stained IgG, IgM, IgA, IgD and IgE. The majority of plasma cells expressed

IgG, followed by IgA, and then IgM and IgE. Compared to IgE producing cells observed in OFG biopsies under allergic condition, there were not many IgE+ cells in GPA biopsies. No cytoplasmic IgD expression was observed in GPA biopsies (Figure 3.2.4).

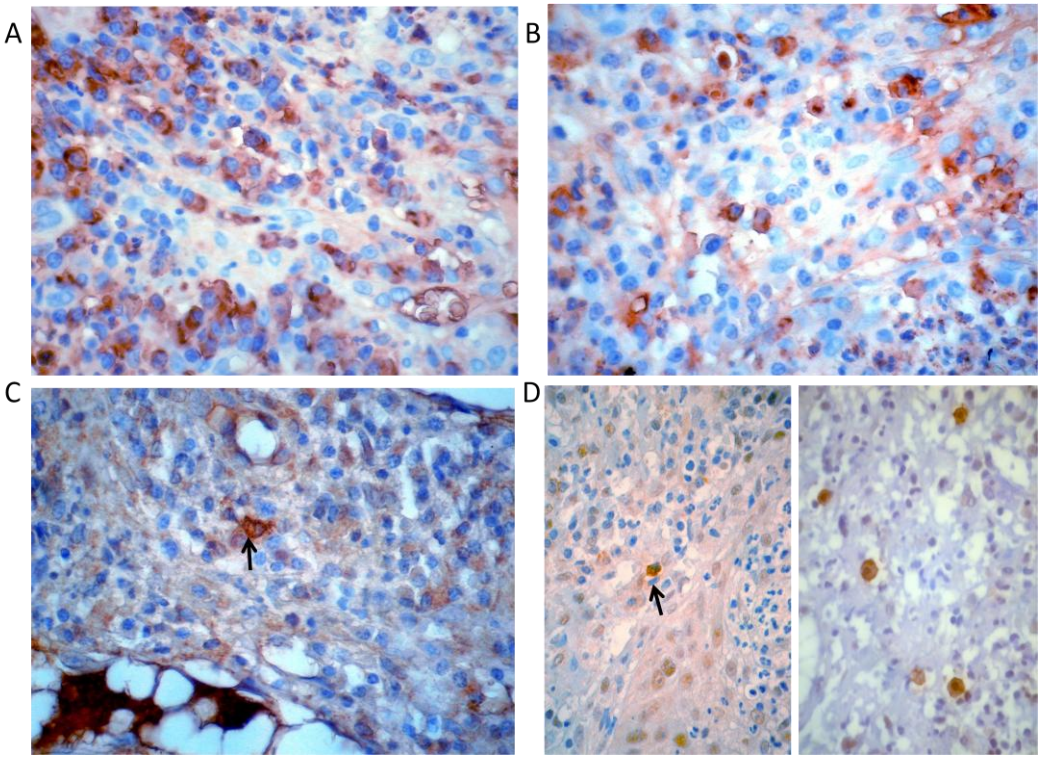

Figure 3.2.4: Examples of Ig productions in paraffin sections of GPA mucosa studied by immunohistochemistry. A. Single staining of IgG (brown) identified IgG producing cells in GPA biopsies, original magnification: x400. **B.** Single staining of IgA (brown) identified IgA producing cells in GPA biopsies, original magnification: x400. **C.** Single staining of IgM (brown) identified IgM producing cells in GPA biopsies (example is pointed out by arrow), original magnification: x400. **D.** Left: Single staining of IgE (brown) identified IgE producing cells in GPA biopsies (arrow points out an example); Right: Single staining of IgE (brown) in OFG biopsies, original magnification: x400.

3.2.2 Study of local B cell clonality in biopsies from GPA patients

Since one patient with GPA donated surplus tissue following surgical procedures in 2004, 2009 and 2010 and a blood sample, IGH gene was studied across these biopsies and blood sample. DNA was prepared from biopsies. Ten PCR reactions to amplify IGH from FR3 to J were carried out from each of the biopsies. PCR products were cloned and sequenced. Clones with the same junctional sequence from the same PCR were considered to be the same sequence and were counted as one. A total of 74 different rearrangements were identified; 40 from bronchial mucosa 2004, 13 from bronchial 2009 and 21 from nasal mucosa 2010. The different numbers were a reflection of different B cell numbers in the different sized tissue samples studied. Clone specific primers were then prepared to the V proximal N-D sequence to specifically identify the clone in target DNA sample by PCR. Design ensured that some non-templated sequence was present between the clone specific primer and the J segment primer to allow confirmation of clonal identity by sequencing. Of 14 primers designed, 8 were found not to amplify non-specifically in a DNA preparation of tonsil cells from an unrelated individual and were considered to be potentially clone specific. When PCR products of the correct size were generated by clone specific PCR, clonal identity was confirmed by sequencing.

Figure 3.2.5: Immunoglobulin heavy chain VDJ junction sequences from a patient with GPA. **A and B.** Alignments of sequences of VDJ obtained from different DNA samples of a biopsy obtained in 2004. The presence of the same sequence in different PCRs confirms B cell division and local presence of members of a B cell clone. **C.** One VDJ rearrangement was identified in both the 2004 and the 2009 biopsies from this patient, implying that this B cell clone was persistent in the lesion for at least 5 years.

Although the B cell population was polyclonal, these methods identified three examples of B cell clonal expansion in the inflamed mucosa. Two different sequences were found to be present from different PCRs in the 2009 biopsy (Figure 3.2.5 A and B). Remarkably, one clone was observed to be present in bronchial tissue in both 2004 and 2009 (Figure 3.2.5 C).

The same method was also applied to detect circulating B cell subsets (details described in section 3.2.4) isolated from blood, using primers designed to the CDR3 sequences observed in the biopsy taken from the patient at the same time.

However, no evidence was observed that B cell clones identified in the affected mucosa circulated in peripheral blood in any sorted subsets.

3.2.3 Study of local production of B cell survival factors in biopsies from GPA patients

Since local B cell division and sustained clonal expansion was found in GPA biopsies as described above, the local distribution of B cell survival factors APRIL and BAFF and the BAFF receptor was investigated by IHC staining. B cells expressing the BAFF receptor and cells producing BAFF were identified in the mucosa in GPA indicating that this pathway of promoting B cell survival is also relevant to the local response in GPA (Figure 3.2.6).

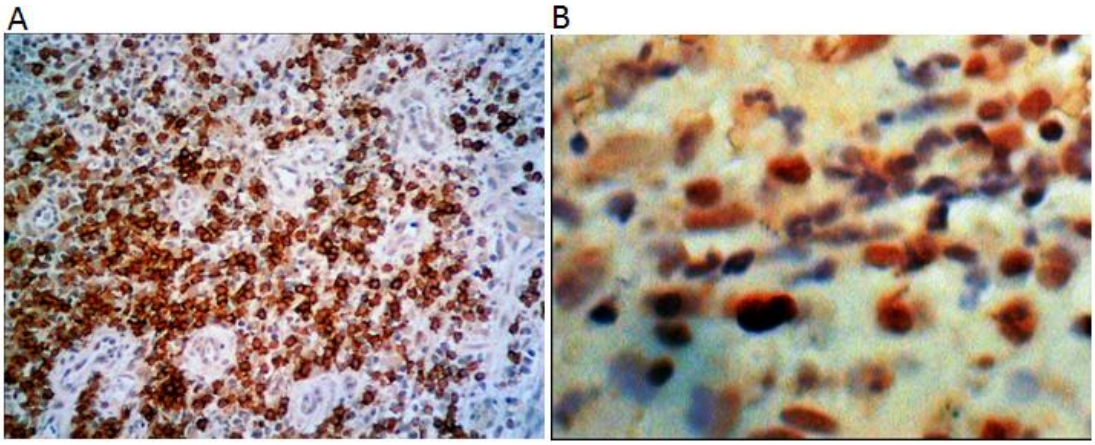

Figure 3.2.6: Examples of BAFF and BAFF-R in paraffin sections of GPA mucosa studied by immunohistochemistry. **A.** Single staining of BAFF-R (brown) identified the distribution of B cells that expressed receptor for survival factor BAFF in biopsies from GPA patients, original magnification: x400. **B.** Single staining of BAFF (brown) identified the production of B cell survival factor BAFF in GPA, original magnification: x600.

APRIL was found to be produced abundantly in the B cell microenvironment in GPA and in other control mucosal biopsies. It was observed that granulomas and giant cells, both of which are features of most cases of GPA, contained abundant APRIL (Figure 3.2.7 A). Granulomas in orofacial granulomatosis and sarcoid were also found to produce APRIL (Figure 3.2.7 B and C). Granulomas in GPA are therefore a previously unrecognized source of APRIL that may contribute to the support of the local B cell response.

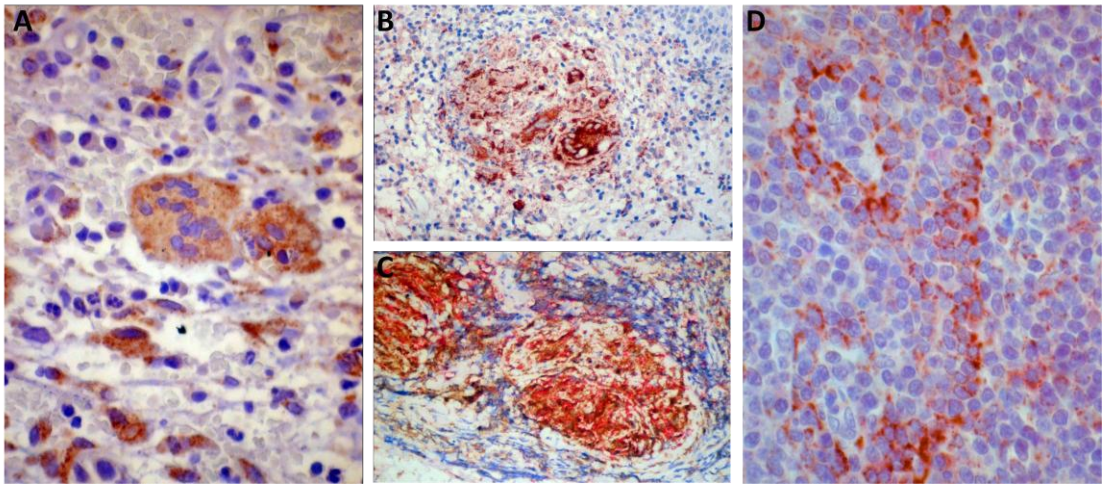

Figure 3.2.7: Examples of APRIL in paraffin sections of GPA mucosa and controls studied by immunohistochemistry. **A.** single staining of APRIL (brown) illustrated the B cell survival factor APRIL production in GPA. Giant cells producing APRIL were observed in GPA, original magnification: x400. **B.** Single staining of APRIL (brown) in OFG. A granuloma containing a giant cell producing APRIL is shown here, original magnification: x200. **C.** Double staining of APRIL (brown) and CD68 (pink) illustrated the APRIL was produced by the macrophages that comprise the granulomas in sarcoidosis, original magnification: x200. **D.** single staining of APRIL (brown) in tonsil section, original magnification: x400.

3.2.4 Study of circulating B cells expressing different regional homing receptors in peripheral blood from GPA patients

Lymphocyte subsets can localize to different tissues by expressing different combinations of adhesion molecules and chemokine receptors. The possibility that the potential lymphocyte homing patterns may be distracted in GPA was investigated. Previous studies have illustrated that cells expressing α4β7 tend to migrate to gastrointestinal mucosa [205]. Other studies indicated a potential role of CLA and CCR10 in guiding cells localize in skin [209, 211]. Table 3.2.1 that is adapted from a previous review [205] describes combination of adhesion molecules and chemokine receptors that would contribute to lymphocytes migration to skin and airway mucosa. Cells co-expressing both the antigen CLA that binds the endothelial antigen E-selectin, and the chemokine receptor CCR10 have the potential to re-localize to skin. Cells expressing only CLA but not CCR10 tend to home to nasal and oral mucosa, while cells expressing CCR10 but not CLA tend to localize in bronchial mucosa. Therefore, B cells expressing these molecules in peripheral blood from GPA patients were studied to investigate whether the chronic expansion of B cells in the mucosa was associated with any changes in the proportion of total lymphocytes or B cells in blood.

Table 3.2.1: Regional homing receptors involved in guiding lymphocyte migration from human peripheral blood into target tissues in GPA

Potential homing site	α4β7	CCR10	CLA
Oral mucosa	-	-	+
Skin	-	+	+
Bronchi	-	+	-

Staining in biopsies from GPA patients indicated the expression of E-selectin by flat endothelium in mucosa (Figure 3.2.8 A). Double staining of E-selectin and CLA in biopsies from GPA indicated CLA+ cells interact with E-selectin expressing vessel endothelium in GPA biopsies (Figure 3.2.8 B). This supports the concept that cells expressing CLA might migrate from blood to lesions in GPA patients.

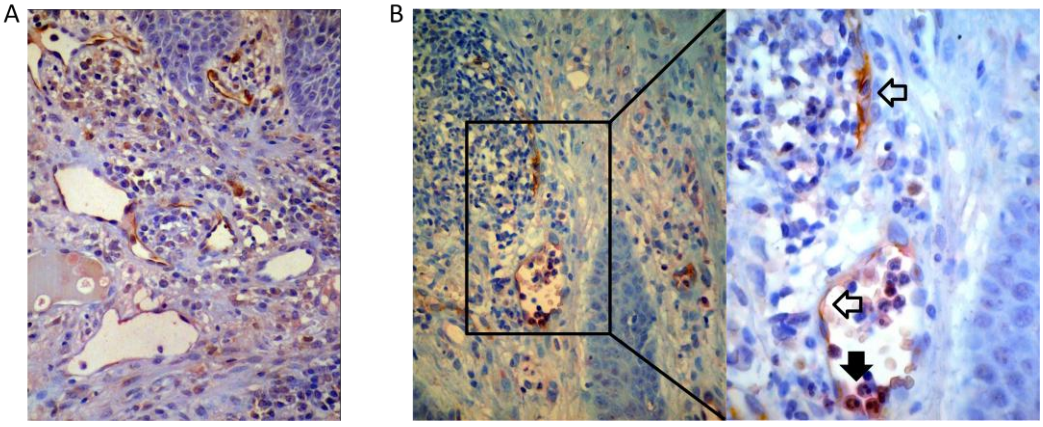

Figure 3.2.8: Examples of E-selectin and CLA in paraffin sections of GPA mucosa and controls studied by immunohistochemistry. **A.** Single staining of E-selectin (brown) illustrated E-selectin expression in GPA mucosa, original magnification: x200. **B.** Double staining of E-selectin (brown, pointed out by open arrow) and CLA (pink, pointed out by closed arrow) illustrated CLA+ cells interact with E-selectin expressing vessel endothelium, original magnification: x200 (left), x400 (right).

Therefore cells expressing CLA, either amongst B cells or in the total lymphocyte in peripheral blood from 16 GPA patients and 11 age and gender matched healthy controls were studied by flow cytometry. Patients' clinical information is shown in table 3.2.2. Gating strategy is shown in figure 3.2.9.

Table 3.2.2: Clinical information of GPA patients

		Gender	Age	Disease duration (yrs)	Ab	BVAS	Organ involvement	ISD
GPA	P1	M	66	5	cANCA	4	sinuses, orbit, renal,skin, joints	MTX, pred
	P2	F	69	10	cANCA	0	sinuses, nose, ear	Pred, MTX
	P3	M	37	5	cANCA	12	sinuses, orbit, renal, nerves, lungs	Pred
	P4	F	43	not known	cANCA	10	sinuses,orbit, lungs, ears	MMF, Pred
	P5*	F	40	4~5	cANCA	3	lungs	Pred
	P6	M	47	<1	cANCA	4	lungs	Pred
	P7	F	53	4	cANCA	6	sinuses, renal	AZA, Pred
	P8	F	66	not known	neg	2	sinuses, nerves, ears, nose, throat	Pred
	P9	M	63	8	neg	5	sinuses,orbit,skin	MTX, Pred
	P10	M	63	6	cANCA	9	sinuses, orbit, nerves	MTX, Pred
	P11	F	60	13	cANCA	8	sinuses, orbit, nerves, skin	AZA, Pred
	P12	F	38	not known	cANCA	6	sinuses	NA
	P13	M	25	<1	cANCA	36	polyarteritis nodosa	CYC
	P14	F	55	3~4	cANCA	13	renal, nerves, trachea, nose	MTX, Pred
	P15	M	28	3	cANCA	3	sinuses, orbit, lungss, ears	AZA
	P16	M	47	1.5	neg	5	sinuses, orbit, trachea, lungs, ear, nose	NA

* IgH chain gene analysis used to study biopsies and blood sample.

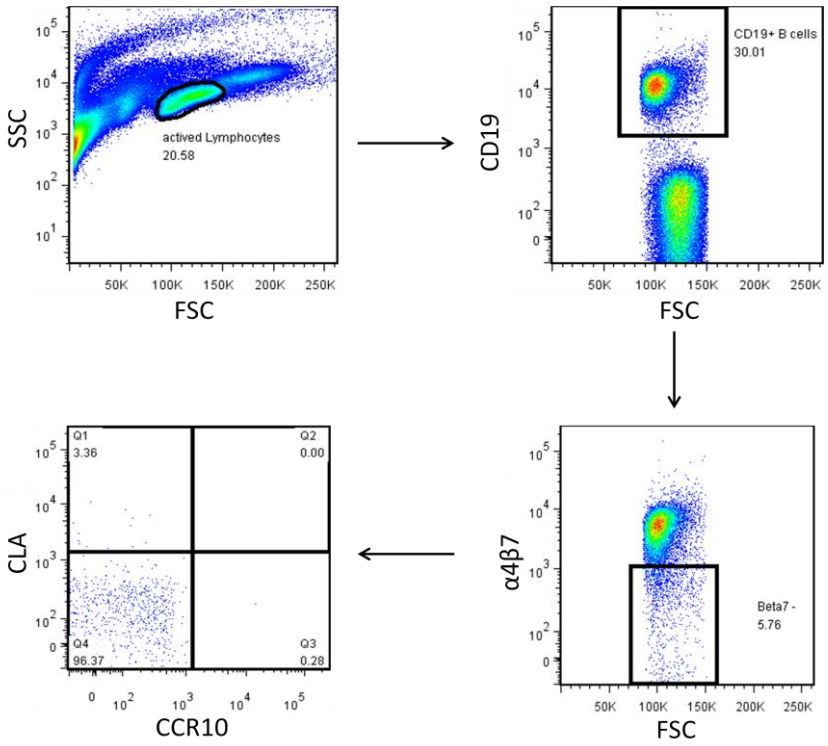

Figure 3.2.9: Gating strategy of circulating B cells expressing different homing receptors in peripheral blood from GPA patients and healthy controls.
Lymphocytes identified by their forward and side scatter properties were gated for CD19+ expression and then examined for negative expression of α4β7, and then studied for CLA and CCR10 expression.

Firstly, the frequencies of CD19+α4β7- B cells, which could potentially migrate to non-gastrointestinal mucosa, in total circulating CD19+ B cells from GPA patients and healthy controls were investigated. No difference in the percentage of α4β7- B cells in total B cells was found between GPA patients and healthy controls (Figure 3.2.10).

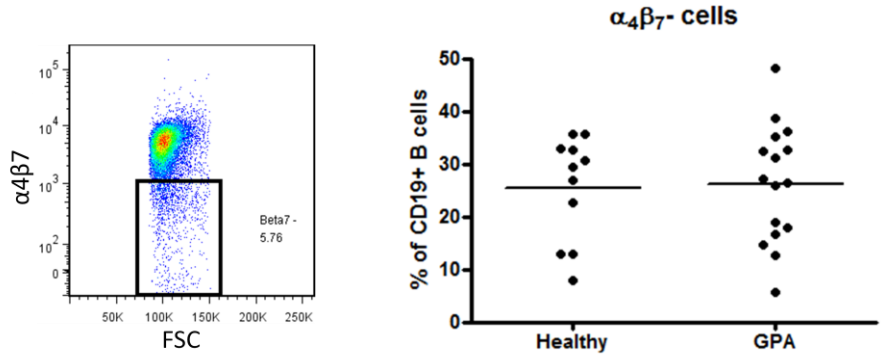

Figure 3.2.10: Analysis of α4β7- cells in CD19+ B cells in peripheral blood from GPA patients and healthy controls. **A.** Gating strategy of α4β7- cells in CD19+ B cell population. **B.** Percentage of α4β7- cells in CD19+ B cells in peripheral blood from GPA patients (average: 26.29, range: 5.76 to 48.1) and healthy controls (average: 25.5, range: 7.8 to 35.72) (P=0.94).

Further study found no difference in the proportion of CLA+CCR10- cells in CD19+α4β7- B cell population between GPA patients (average: 8.19, range: 0.51 to 20.38) and healthy controls (average: 6.6, range: 1.79 to 15) (Figure 3.2.11, P=0.57).

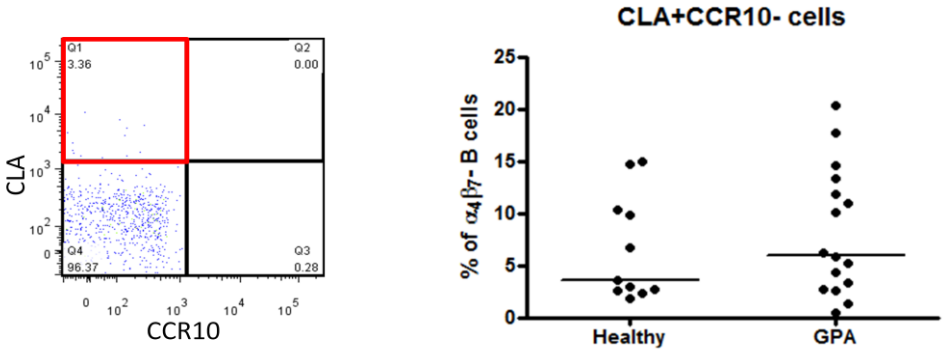

Figure 3.2.11: Analysis of CLA+CCR10- cells in α4β7- B cells in peripheral blood from GPA patients and healthy controls. **A.** Gating strategy of CLA+CCR10- cells in α4β7- B cell population. **B.** Percentage of CLA+CCR10- cells in α4β7- B cells in peripheral blood from GPA patients (median: 6.01, range: 0.51 to 20.38) and healthy controls (median: 3.57, range: 1.79 to 15) (P=0.57).

There were very few CLA and CCR10 double positive cells observed in the CD19+α4β7- B cell population. No difference was found between GPA patients (average: 0.025, range: 0 to 0.2) and healthy controls (average: 0.0055, range: 0 to 0.05) (Figure 3.2.12, P=0.49).

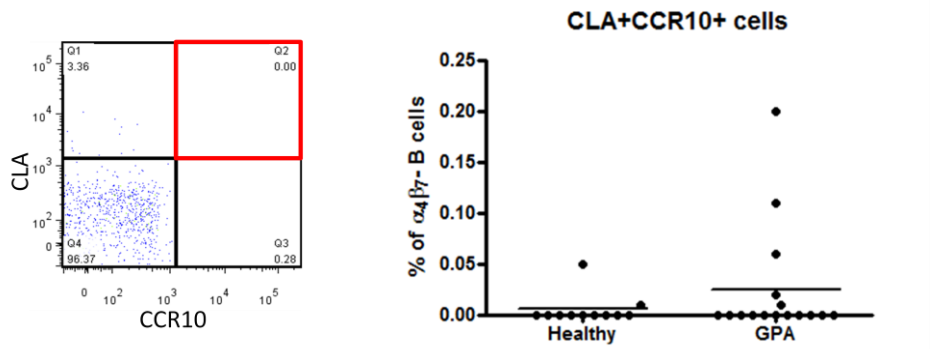

Figure 3.2.12: Analysis of CLA+CCR10+ cells in α4β7- B cells in peripheral blood from GPA patients and healthy controls. **A.** Gating strategy of CLA+CCR10+ cells in α4β7- B cell population. **B.** Percentage of CLA+CCR10+ cells in α4β7- B cells in peripheral blood from GPA patients (average: 0.025, range: 0 to 0.2) and healthy controls (average: 0.0055, range: 0 to 0.05) (P=0.49).

No difference in the proportion of B cells expressing CCR10 but not CLA in the CD19+α4β7- B cell population was observed between patients with GPA (average: 0.57, range: 0 to 5.3) and healthy controls (average: 0.25, range: 0 to 0.42) (Figure 3.2.13, P=0.96).

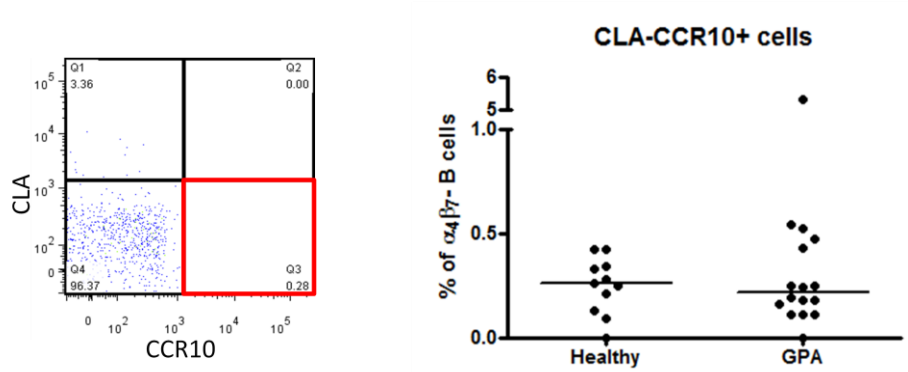

Figure 3.2.13: Analysis of CLA-CCR10+ cells in α4β7- B cells in peripheral blood from GPA patients and healthy controls. **A.** Gating strategy of CLA-CCR10+ cells in α4β7- B cell population. **B.** Percentage of CLA-CCR10+ cells in α4β7- B cells in peripheral blood from GPA patients (median: 0.22, range: 0 to 5.3) and healthy controls (average: 0.25, range: 0 to 0.42) (P=0.96).

CLA could be expressed on other cell types. Accumulation of CLA+ T cells has been reported to be associated with various skin diseases [210]. The percentage of CLA+ cells in total lymphocytes from GPA patients and healthy controls were therefore studied. However, as shown in figure 3.2.14, no difference was found in the proportion of CLA+ cells in total lymphocytes between GPA patients (average: 10.32, range: 1.37 to 19.6) and healthy controls (average: 9.45, range: 2.7 to 19.04) (P=0.54).

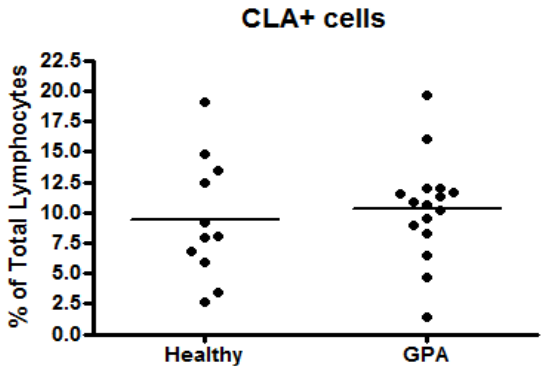

Figure 3.2.14: Analysis of CLA + cells in total lymphocytes in peripheral blood from GPA patients and healthy controls. Percentage of CLA+ cells in total lymphocytes in peripheral blood from GPA patients (average: 10.32, range: 1.37 to 19.6) and healthy controls (average: 9.45, range: 2.7 to 19.04) (P=0.54).

3.2.5 Main findings in this chapter

- B cells and plasma cells surrounded by PR3 expressing cells and abundant B cell survival factors were present in biopsies from GPA patients.
- Approximately 5% of those B cells were observed dividing in lesions from GPA patients.
- B cell clonal expansion was observed in biopsies from GPA patients through analysis of IgH gene.
- B cell clonity was observed to be persistent in the bronchial lesion for five years in one GPA patient.
- No evidence of B cell clones presented in lesions circulating in peripheral blood was observed in GPA patients.
- No difference in the percentages of circulating B cell subsets that have potential to migrate to skin and non-gastrointestinal mucosa in GPA patients was observed.

3.3 Discussion

3.3.1 Microenvironment in lesions from GPA

The microenvironment in biopsies from GPA patients has been described in this chapter. Chronically activated B cells and PR3 expressing cells have been observed in biopsies from GPA patients. These findings are consistent with previous studies of patients with GPA [317, 325].

Inflamed mucosa containing chronically activated B cells intimately mixed with cells expressing PR3, a characteristic target of autoantibodies, has been observed in most biopsies from GPA patients in this thesis. The most common Ig isotype observed in biopsies from GPA patients was IgG, consistent with the profile observed in the diverse set of control tissues. Granuloma is a common feature present in both GPA and OFG patients [3, 364]. Similar to the subepithelial dendritic B cells identified recently in oral mucosa from OFG patients [358], B cells observed in lesions from GPA patients were activated cells that lack IgD. Consistent with a previous study [323], proliferating B cells were found to compose approximately 5% of those B cells in biopsies from GPA patients. Moreover, these B cells were observed to have irregular cytoplasmic processes that extend and contact diverse adjacent cells. They are not necessarily associated with any follicular structures but exist alongside a plasma cell component in inflammation. Such cells are not known to be a general feature of the physiological process of plasma cell differentiation from B cells. For

example the lamina propria of the normal gut, a site of plasma cell differentiation, does not have a significant population of proliferating cells that resembles those observed in the mucosa described here.

Abundant expression of B cell survival factors APRIL and BAFF in biopsies from GPA patients has been described in this chapter. BAFF and APRIL have been reported to support B cell survival [94, 103]. Previous studies have reported increased levels of plasma and serum BAFF, but no difference in serum APRIL level in peripheral blood from GPA patients [361-363]. The relevance of serum BAFF to ANCA status and vasculitis remains unclear, in a study of GPA serum BAFF varied inversely with ANCA levels [361, 362]. Studies have not observed any correlation between concentrations of APRIL in serum and ANCA titers [363]. Animal studies have demonstrated that BAFF plays an important role in regulating B cell maturation during transitional checkpoint [108]. Other studies have indicated that over-expression of BAFF could lead to autoimmune symptoms reminiscent of SLE and Sjogren syndrome [360]. Therefore the abundant expression of BAFF and APRIL observed in lesions from GPA patients may contribute to the survival of B cells and local auto-antibody production in GPA. It is not known if BAFF and APRIL produced in mucosa acts locally or if it may contribute to the serum pool of B cell survival factors.

In addition to supporting B cell survival, BAFF has also been reported to support T cell proliferation through interaction with BAFF-R [116, 117]. Therefore

BAFF production observed in biopsies from GPA patients may contribute to the pathogenesis of the disease by multi-pathways.

Giant cells have been observed in the lesions of the respiratory tract from patients with GPA [356]. The expression of APRIL by granulomas and giant cells has been described in this thesis for the first time. It is possible that the granulomas, which are also linked to macrophage fusion and giant cell formation, may subsequently provide a source of factors to support an aberrant B cell response. This observation is relevant to granulomatous diseases other than GPA and is therefore not restricted to this autoimmune condition. However, the combination of mixed acute and activated chronic inflammatory cells, alongside survival factors including APRIL produced by granulomas and giant cells was only seen in GPA.

3.3.2 B cell clonality study in biopsies from GPA patients

The B cell clonality in different biopsy sections donated at different time points and paired blood sample from one GPA patient has been studied in this chapter.

Consistent with a previous study [323], clonally related cells were detected in a single bronchial biopsy section in a polyclonal B cell background by comparison of Ig gene CDR3 sequences, implying local B cell clone expansion. This finding

is also supported by the observation of proliferating B cells by immunohistochemistry in this thesis.

B cell clonality in biopsies of the same bronchial lesion from one patient collected for diagnosis at different time points has been described for the first time in this thesis. A clonal population of B cells has been found to be present in two biopsies obtained from the same site in one GPA patient in 2004 and 2009 respectively. This presence of the B cell clone persisting for 5 years in one patient suggests that B cell stimulation in GPA is chronic. In this chapter, the B cells in biopsies from GPA patients were observed to be often intimately associated with cells expressing PR3. Previous studies have reported PR3 expression in lesions from GPA patients [317]. Consistent with this study, some of the PR3 positive cells observed in this thesis were clearly neutrophils, but not all. It is possible that the abundance of PR3 antigen locally provides an immunological stimulus to potentially initiate or sustain the chronic B cell anti-PR3 response. However some cases studied in this thesis were negative for autoantibodies to PR3. This demonstrates that although the presence of PR3 positive cells adjacent to B cells in lesions of GPA could possibly support the generation of autoantigen reactive B cells, this does not necessarily occur. A population of neutrophils that support B cell responses has recently been described suggesting that neutrophils activating B cell responses may be involved in pathogenesis of GPA [365].

Consistent with previous studies [323, 325], the IGH sequences obtained from biopsies from GPA patients contained mutants which generated by somatic hypermutation (SHM) (data not shown). In normal condition, this suggested that these B cells were driven from germinal centres. However, although CD3+ T cells and CD20+ B cells were observed in adjacent area in GPA biopsies, germinal centres were not found as a common feature in GPA mucosa. It has been reported that B cell responses that result in germinal centre formation are generally T cell dependent [59]. T cell involvement places stringent regulation on the discrimination between self and non-self as a consequence of thymic education [158, 222]. However, in most cases studied in this thesis, no organized lymphoid structure was observed in GPA. There is one study reporting germinal centre-like structures and also diffusely infiltrating lymphocytes in granuloma lesions from GPA [326]. Previous studies on mice have demonstrated that CSR in BAFF-transgenic mice is T cell independent [359]. Further studies on human blood and mice demonstrated that BAFF and APRIL could induce CSR [95, 114]. Therefore the chronic B cell activity but lack of germinal centre formation in most disease relevant mucosal microenvironments in GPA might indicate that the anti-PR3 response in the mucosa could be driven in a T cell independent way.

Moreover, the mutants found in IGH gene in lesions from GPA patients might be induced by the presence of abundant BAFF and APRIL in the microenvironment but not from a local germinal centre response.

3.3.3 Study of circulating lymphocytes that may migrate to upper airway lesions in GPA

The B cells in mucosal lesions in GPA have been observed to have mutated IgHV genes, yet in the majority of cases no GC formation was observed in the mucosa. We therefore considered that the B cell fraction that localizes to the mucosa might be expanded as a population in blood and lymph nodes in GPA as part of a migratory continuum. It is also possible that recruitment to an inflammatory site might result in depletion of a subset of inflammatory cells from the blood. When we analyzed the subsets of total lymphocytes or B cells in blood that expressed markers associated with tissue specific homing, no differences between GPA and healthy controls were apparent.

Although other studies have observed that CD4+CD45RO+CCR7- T cells disappeared from peripheral blood but appeared in lesions in patients with activated GPA [314], no difference was observed in subsets of B cells and T cells that might be guided to migrate towards skin and non-gastrointestinal mucosa in GPA patients in this thesis. In addition, no evidence of that B cell clones identified in biopsies were circulating in the paired peripheral blood in GPA patients was found through IgH gene study in this thesis. These data together suggest that B cells in the lesion from GPA patients would be not a component of circulating B cells in the peripheral blood. This hypothesis is supported by one

published study reporting B cell presence in lesions from rituximab treated GPA patient when B cells in peripheral blood has been depleted [333].

3.3.4 Conclusion

- Chronic activated B cells observed in lesions from GPA patients could be stimulated by sustained PR3 expression and supported by local production of BAFF and APRIL.
- B cell clonal expansion in the lesion of GPA might be a result from T cell independent response but supported by abundant BAFF and APRIL in the micro-environment as no organized lymphoid structure was observed in most cases.
- B cells present in the lesion might not be a component of any circulating B cell subsets that might have the potential to migrate to the target tissue as no evidence was found in B cell clonality study and the study of circulating B cell subsets.

Chapter 4

Study of circulating T_{FH} cells and Treg cells in GPA

4.1 Introduction

Rituximab is a chimeric monoclonal antibody against B cell surface marker CD20 [330] that is used as a B cell depletion therapy to treat different autoimmune diseases [366]. Although some studies have reported that rituximab lacks efficiency in treating patients with GPA [367, 368], rituximab is considered to be a beneficial therapy for GPA patients by many clinicians [369-371]. Recently, rituximab was approved for use in GPA patients by the Food and Drug Administration in the USA, based on two successful non-inferiority randomized controlled clinical trials [23, 24]. Although the pathology in autoimmune diseases may be autoantibody mediated, it is considered unlikely that disease remissions achieved by rituximab are a consequence of autoantibody depletion alone [372]. The relative importance of B cells and T cells in the pathogenesis of autoimmune diseases has been debated. Whilst autoimmune rheumatic diseases are consistently linked with the production of autoantibodies, implicating B cell involvement, T cells are generally associated with the generation of autoimmune pathology [222]. The success of B cell depletion therapy in alleviating the symptoms of autoimmune diseases has highlighted the importance of B cells [339, 373-375]. There is increasing evidence from animal models and patient studies indicating that rituximab treatment also influences T cell subsets which directly affect disease development. Studies on the EAE mouse model identified a decreased frequency of CD4+ T cells in spleen and

lymph nodes from rituximab treated mice [376]. Other studies on SLE patients following B cell depletion therapy demonstrated down-regulated CD40L on the T cell surface [377], increased Th1 but not Th2 cells [378].

Certain T cell subsets may be intrinsically dependent on T-cell dependent B-cell responses. T follicular helper (T_{FH}) cells, for example, are a T cell subset that normally resides in the germinal centres in the B cell zones of secondary lymphoid tissues [59]. Clones of T_{FH} cells are known to disseminate widely and to circulate as a minority subset of T cells in the blood [150]. In human peripheral blood, CD4+ T cells expressing CXCR5, which is receptor for chemokine CXCL13 produced in the germinal centres, were found to be able to migrate to B cell follicles [151]. Other studies subdivided the CD4+CXCR5+ T cell population into Th1, Th2 and Th17 cells and indicated that Th2 and Th17 can provide help to naïve B cells to differentiate and produce Abs [379]. Although circulating CD4+ CXCR5+ T cells in peripheral blood may have similar functions as T_{FH} cells from germinal centres, the relationship between those cells is not clear yet. Whereas T_{FH} cells are defined by their microanatomical location in the germinal centres in tissues, a small subset of CD4+ T cells in human blood was described as circulating T_{FH} cells by their high levels co-expression of CXCR5 and cell surface antigen PD-1 [158].

Germinal centre T_{FH} cells are involved in the progression of autoimmunity in a mouse model of SLE by lowering the threshold for B cell survival [222]. This

lack of stringency in T cell mediated B-cell selection supports the evolution of autoreactive antibody secreting clones of cells. There is evidence that T_{FH} cells may be involved in autoimmune responses in humans in a subset of patients with severe SLE, where the proportion of circulating T_{FH} cells, which were defined as CXCR5+CD4+ T cells expressing high levels of ICOS or PD-1, was significantly higher than that in healthy controls [158].

Regulatory T (Treg) cells are critical regulators of autoimmune diseases through suppressing the proliferation and cytokine production of effector T cells [334, 335]. The failure of Treg cells to suppress inflammation, either through defects of Treg cells or the cells that respond to them, may facilitate the development of autoimmune pathology. Some studies have shown Treg cell frequencies to be reduced in GPA, and other studies have detected defects in the suppressive function of Treg cells in GPA - both sets of observations implicate Treg cells in the disease process [336-338]. Interestingly, Treg cell frequencies have been shown to increase in the blood of patients with SLE following rituximab treatment [339]. This could be a secondary consequence of clinical remission, or if Treg cells are actually associated with the disease process and are not purely bystanders, this data might imply that Treg cells are in some way B cell dependent.

The pathogenesis of GPA is not well understood. Although GPA patients have benefited from B cell depletion therapy with rituximab, the mechanism of

remission is not clear. The effects of B cell depletion on different T cell subsets in GPA patients following rituximab therapy have not been studied. Therefore circulating T_{FH} cells and Treg cells from GPA patients following B cell depletion therapy with rituximab, GPA patients on conventional therapies, healthy and disease controls were studied in this chapter.

Aims of this chapter:

The aim of this chapter is to investigate any difference in frequencies of circulating T_{FH} cells and Treg cells from GPA patients on conventional therapies, B cell depleted GPA patients, healthy and disease controls.

4.2 Results

4.2.1 Study of frequencies of B cells and T cells in peripheral blood from GPA patients

4.2.1.1 Frequencies of B cells in peripheral blood from GPA patients, B cell depleted GPA patients and healthy controls

CD19 expression in PBMC from 18 GPA patients on conventional therapies, 9 GPA patients following B cell depletion therapy and 11 age and gender matched healthy controls were studied by flow cytometry and data was analysed. As shown in figure 4.2.1, the proportion of CD19+ B cells in GPA patients following B cell depletion therapy (average: 0.68) was significantly lower than in GPA patients on conventional therapies (average: 15.89) ($P<0.0001$) and healthy controls (average: 13.39). There was no difference in the frequency of CD19+ B cells in peripheral blood between GPA patients on conventional therapies and healthy controls.

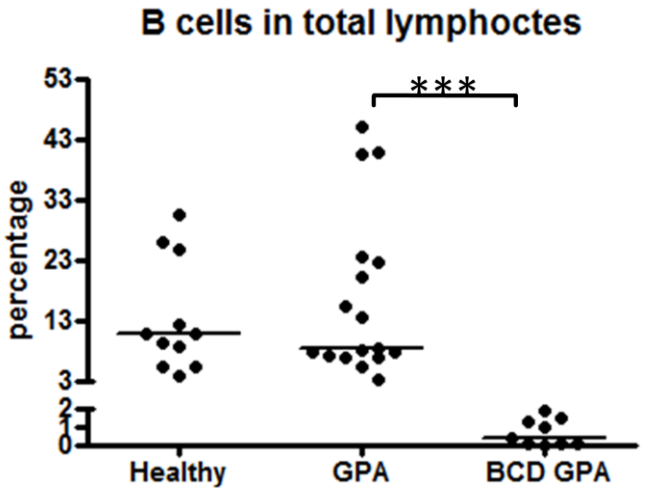

Figure 4.2.1: Frequencies of B cells in total lymphocytes from GPA patients on conventional therapies, B cell depleted GPA patients and healthy controls. Percentage of CD19+ B cells in total lymphocytes from healthy controls (median: 10.64, range: 3.75 to 30.36), GPA patients on conventional therapies (median: 8.26, range: 2.9 to 45.08) and GPA patients under B cell depletion therapy (average: 0.68, range: 0 to 1.9) ($P<0.0001$).

4.2.1.2 Clinical effect of B cell depletion therapy on GPA patients

Since there were few B cells in the peripheral blood from GPA patients following RTX therapy, the clinical effect of B cell depletion therapy on GPA patients was studied by comparing the BVAS scores before and after RTX treatment in the same patient cohort. Clinical information of GPA patients on conventional therapies is shown in table 4.2.1.

Table 4.2.1: Clinical information of GPA patients on conventional therapies:

		Gender	Age	Disease duration (yrs)	Ab	BVAS	Ab titers		Organ involvement	ISD	
							PR3	MPO		Previous	Current
GPA	P-1	M	63	6	cANCA	9	17.9	0	sinuses, eyes, nerves, ears	MTX, Pred	MTX, Pred
	P-2	F	60	13	cANCA	8	16.8	0	Sinuses, ears, lungs, nerves, skin	AZA, Pred	AZA, Pred
	P-3	F	38	>2	pANCA	13	0.5	0	sinuses, nerves	AZA, MTX, Pred	DECLINED
	P-4	M	25	<1	cANCA	30	212	85	sinuses, orbit, renal, nerves, trachea, lungs	CYC, Pred	AZA, Pred
	P-5	F	55	3~4	cANCA	13	0.4	3.4	renal, ears, nose, trachea, nerves	MMF, AZA, Pred	MTX, Pred
	P-6	M	28	3	cANCA	3	13.8	0	ears, eye, trachea	AZA, Pred	AZA, Pred
	P-7	M	47	1.6	neg	5	0	0	eyes, ears, nose	MTX, Pred	MTX, Pred
	P-8	M	51	3	cANCA	4	36.5	0	sinuses, renal, skin, lungs	MTX, Pred	MTX, Pred
	P-9	F	36	14	cANCA	6	17	0	sinuses, trachea, skin, eyes	AZA, CYC, Pred	AZA, Pred
	P-10	M	41	15	cANCA	6	18.3	0	eyes, ears, renal, lungs	CYC, RTX, MMF, Pred	MTX, Pred
	P-11	F	61	23	cANCA	21	25.9	0	lungs, renal, nerves	CYC, AZA, Pred	AZA, Pred
	P-12	F	37	1	cANCA	6	1.5	1	sinuses, orbit	MTX, CYC, Pred	MTX, Pred
	P-13	F	70	8	neg	3	0	0	sinuses, nerves	MTZ, Pred	MTX, Pred
	P-14	M	49	3	cANCA	19	22.5	0	nose, nerves, brain	AZA, Pred, MMF, MTX, CYC, RTX	Pred
	P-15	M	49	>3	cANCA	8	600	0	eyes, Renal	CYC, MMF, Pred	MMF, Pred
	P-16	F	58	13	neg	12	0	0	ears, nose	AZA, Pred	AZA, Pred
	P-17	F	83	25	cANCA	6	833	0	sinuses, orbit, skin	AZA, Pred	AZA, Pred
	P-18	F	63	>6	cANCA	3	34	0	sinuses, nerves, skin, lungs	MTX, CYC, Pred	MTX, Pred

Table 4.2.2: Clinical information of GPA patients following B cell depletion therapy

		Gender	Age	Disease duration (yrs)	Ab	BVAS		ANCA titers				Organ involvement	Current ISD
						pre-BCD	after-BCD	pre-BCD		after-BCD			
								PR3	MPO	PR3	MPO		
BCD GPA	P-1	M	55	>20	cANCA	18	5	3.6	0.9	2.9	0.9	sinuses, orbit	MTX, Pred
	P-2	M	63	6	cANCA	31	3	264	0.7	10.3	0	sinuses, renal, lungs	AZA, Pred
	P-3	M	55	>20	cANCA	17	2	192	0.2	8.8	0	sinuses, eyes, ears, nerves, lungs	Pred
	P-4	M	54	20	neg	10	3	0	0	0	0	sinuses, ears	AZA, Pred
	P-5	F	73	<1	cANCA	35	7	31.4	3.4	0.9	0	sinuses, ears, renal, lungs, nerves, skin	AZA
	P-6	M	68	10	neg	4	4	0	0	0	0	sinuses, nerves, AI bone marrow suppression	Pred
	P-7	F	46	5	cANCA	4	0	136	1.3	41.9	0	sinuses, ears	MTX
	P-8	F	42	4	cANCA	17	13	8.1	0.6	8.1	0	sinuses, ears, orbit, trachea, lungs, brain	Pred
	P-9	M	65	4	cANCA	15	13	25.1	0.6	0.3	0	sinuses, renal, nerves	CYC oral, Pred

Clinical information of GPA patients following B cell depletion therapy is shown in table 4.2.2, including BVAS scores and antibody titers before and after RTX treatment. 7 out of 9 B cell depleted GPA patients included in this study were ANCA positive. The ANCA titers were found not to change after RTX treatment in 2 out of 7 B cell depleted GPA patients (Table 4.2.2).

All GPA patients following B cell depletion therapy were clinically improved. BVAS scores after RTX treatment were significantly lower than before the treatment (P=0.024) (Figure 4.2.2).

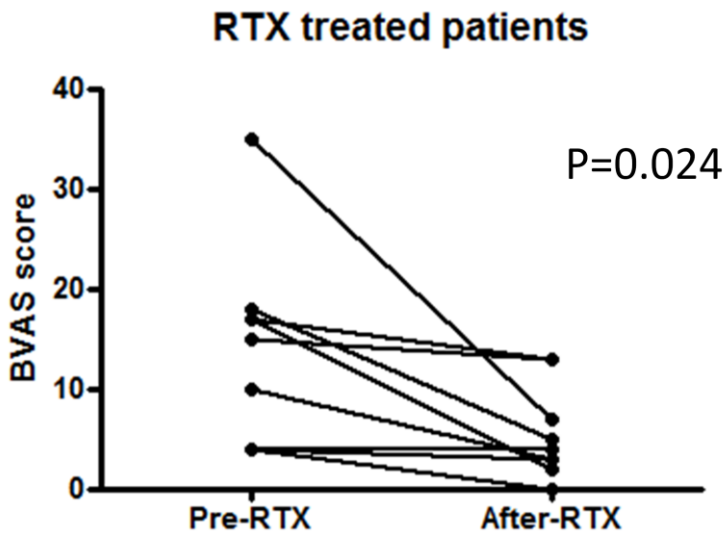

Figure 4.2.2: Disease activity score (BVAS score) change in patients before and after RTX treatment. In the group of patients receiving RTX, BVAS scores were 4 to 35 before RTX, compared to 0 to 13 after RTX (P=0.024).

Blood samples from SLE patients were used as disease controls for study in this chapter. Clinical information of SLE patients studied in this chapter is shown in table 4.2.3.

Table 4.2.3 Clinical information of SLE patients

		Gender	Age	Antibody	Disease duration (yrs)	BILAG SCORES									Organ involvement	Current treatment
						Constitution	Mucocutaneo	Neuropsychiat	Musculoskel	Cardiorespirat	Gastrointestin	Ophthalm	Renal	Haematologic		
SLE	P1	F	34	neg	13	E	D	E	C	E	E	E	E	E	arthritis, mouth ulcers	Hydroxychloroquine, Folic Acid and Multivitamins, Iron supplement
	P2	F	23	ANA, ENA, La	8	C	B	E	C	E	E	E	E	D	arthritis, haematological	Aspirin, Hydroxychloroquine, Tablet Prednisolone, Folic Acid,
	P3	F	46	dsDNA, ANA, aPL	27	D	C	D	C	E	E	E	E	D	malar rash, photosensitivity, arthritis, mouth	Aspirin, Hydroxychloroquine, Tablet Prednisolone, Folic Acid,
	P4	F	23	DsDNA, RO	10	E	B	E	D	E	E	E	E	D	malar rash, photosensitivity,SCL	Aspirin, Hydroxychloroquine, Tablet Prednisolone, Folic Acid,
	P5	F	58	ANA, Ro	>5	E	C	E	C	E	D	E	E	D	Photosensitivity, arthritis, mouth ulcers	Mepacrine, Amlodipine, Diclofenac, Co-codamol.
	P6	F	65	dsDNA, ANA, aPL	9	C	C	B	B	B	E	E	E	E	malar rash, arthritis, mouth ulcers	Aspirin, Hydroxychloroquine, Omeprazole, Calcichew D3,
	P7	F	49	ANA, Ro, La	27	E	C	D	B	E	E	E	E	E	malar rash, arthritis, abdominal pain, nausea, dysphagia	Hydroxychloroquine, Prednisolone, Acitreti, Domperidone, Thalidomide, Methotrexate, Mycophenolate
	P8	F	34	ANA	1	C	C	E	C	B	B	E	E	E	malar rash, photosensitivity, arthritis, mouth ulcers	Mepacrine, Analgesia, PRN

4.2.1.3 Frequencies of T cells in peripheral blood from GPA patients, B cell depleted GPA patients, healthy and disease controls

To investigate the influence of B cell depletion on other types of lymphocytes, the frequency of T cells in peripheral blood from 18 GPA patients on conventional therapies, 9 B cell depleted GPA patients, 10 healthy controls and 7 SLE patients was studied by flow cytometry. The average percentage of CD3+ in total lymphocytes was 69.94% in healthy controls. No significant difference in the proportion of CD3+ T cells in total lymphocytes was observed between all groups of patients (Figure 4.2.3 A). The frequency of CD4+ T cells in CD3+ T cells was further studied. No significant difference in the percentage of CD4+ T cells in CD3+ T cells was found between all groups (Figure 4.2.3 B). Moreover, no difference was observed between all groups when the proportion of CD4+ T cells in total lymphocytes was calculated (Figure 4.2.3 C).

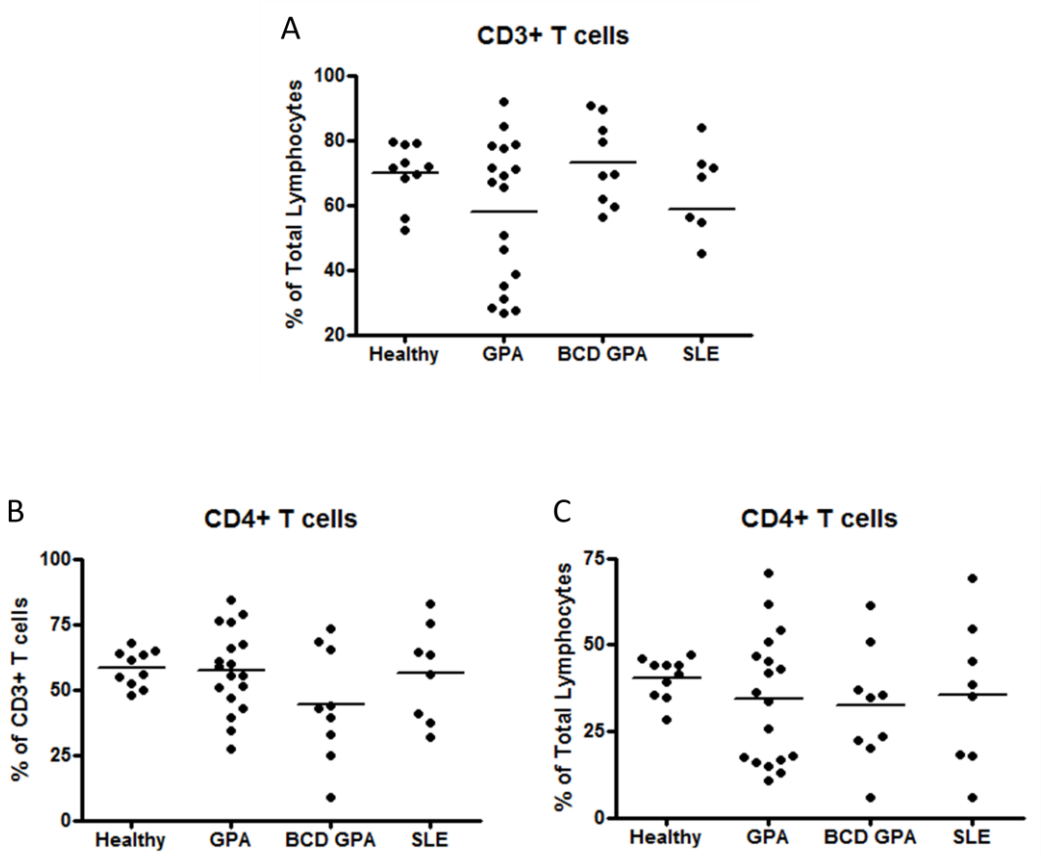

Figure 4.2.3: Frequencies of circulating T cell subsets in peripheral blood from GPA patients on conventional therapies, B cell depleted GPA patients, healthy and disease controls. **A.** Percentage of CD3+ T cells in total lymphocytes in peripheral blood from healthy controls (average: 69.94, range: 52.15 to 79.48), GPA patients on conventional therapies (average: 57.69, range: 26.62 to 92.03), GPA patients under B cell depletion therapy (average: 73.23, range: 56.35 to 90.56) and SLE patients (average: 58.59, range: 16.19 to 83.8). **B.** Percentage of CD4+ T cells in CD3+ T cells in peripheral blood from healthy controls (average: 58.18, range: 47.73 to 67.82), GPA patients on conventional therapies (average: 57.28, range: 27.41 to 84.22), GPA patients under B cell depletion therapy (average: 44.34, range: 8.65 to 73.33) and SLE patients (average: 56.34, range: 31.73 to 82.73). **C.** Percentage of CD4+ T cells in total lymphocytes in peripheral blood from healthy controls (average: 40.49, range: 28.52 to 47.09), GPA patients on conventional therapies (average: 34.28, range: 10.57 to 70.91), GPA patients under B cell depletion therapy (average: 32.38, range: 5.97 to 61.43) and SLE patients (average: 35.58, range: 6.02 to 69.33).

4.2.2 Frequencies of circulating T_{FH} cells in peripheral blood from GPA patients, healthy and disease controls

T_{FH} cells have been shown to be involved in the progression of autoimmunity in a mouse model of SLE by lowering the threshold for B cell survival [222]. Therefore the frequency of circulating T_{FH} cells in peripheral blood from 19 GPA patients on conventional therapies, 9 B cell depleted GPA patients, 10 healthy controls and 8 SLE patients was investigated by flow cytometry. Circulating T_{FH} cells were identified in isolated PBMC as CD3+CD4+ T cells expressing high levels of CXCR5 and PD-1 (Figure 4.2.4).

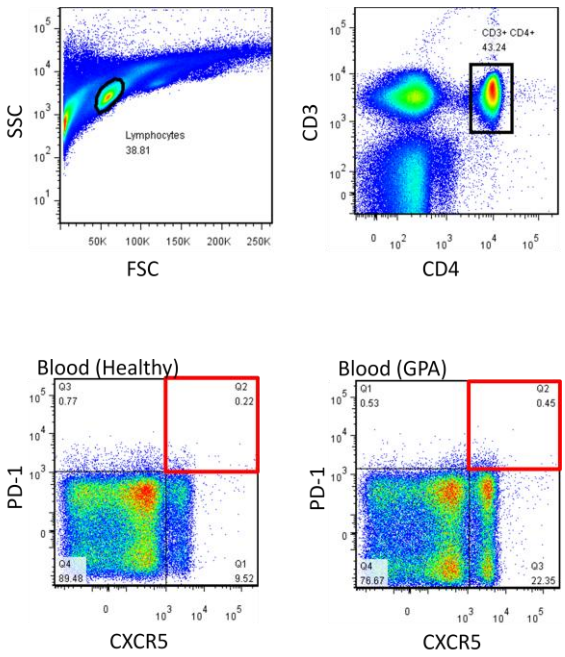

Figure 4.2.4: Gating strategy of circulating T_{FH} cells in peripheral blood from GPA patients on conventional therapies, B cell depleted GPA patients, healthy and disease controls. Lymphocytes identified by their forward and side scatter properties were gated for CD3+ and CD4+ double expression and then examined for high expression of PD-1 and CXCR5 to identify circulating T_{FH} cells in peripheral blood.

A significant increase in percentage of $CXCR5^{high}PD\text{-}1^{high}$ T_{FH} cells in CD4+ T cells was observed in peripheral blood from GPA patients on conventional therapies (average: 0.37) compared to age and gender matched healthy controls (average: 0.19) (P=0. 001) and GPA patients following B cell depletion therapy (average: 0.23) (P=0. 0051). In contrast, no such difference was found between healthy controls and either B cell depleted GPA patients (P=0.5) or SLE patients (average: 0.3) (P=0.27) (Figure 4.2.5 A).

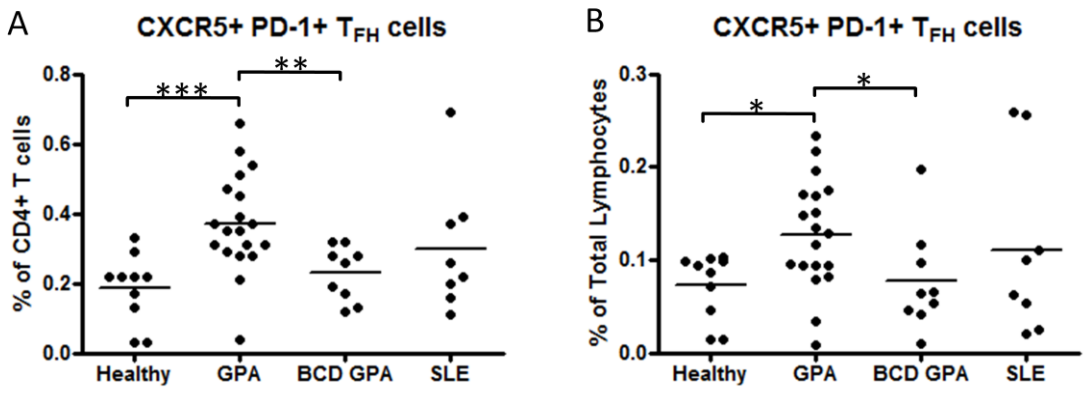

Figure 4.2.5: Frequencies of circulating T_{FH} cells in peripheral blood from GPA patients on conventional therapies, B cell depleted GPA patients, healthy and disease controls. **A.** Percentage of circulating T_{FH} cells in CD4+ T cells in peripheral blood from healthy controls (average: 0.19, range: 0.03 to 0.33), GPA patients on conventional therapies (average: 0.37, range: 0.04 to 0.66) (P=0.001 and P=0.0051 when compared to healthy controls and GPA patients under B cell depletion therapy respectively), GPA patients under B cell depletion therapy (average: 0.23, range: 0.12 to 0.32) and SLE patients (average: 0.3, range: 0.11 to 0.69). **B.** Percentage of circulating T_{FH} cells in total lymphocytes in peripheral blood from healthy controls (average: 0.073, range: 0.014 to 0.1), GPA patients on conventional therapies (average: 0.13, range: 0.0087 to 0.23) (P=0.037 and P=0.044 when compared to healthy controls and GPA patients under B cell depletion therapy respectively), GPA patients under B cell depletion therapy (average: 0.077, range: 0.0096 to 0.2) and SLE patients (average: 0.11, range: 0.021 to 0.26).

As the proportion of CD4+ T cells in total lymphocytes varies between individuals (7.43% to 69.11% in samples studied in this thesis), the frequency of $CXCR5^{high}PD\text{-}1^{high}$ T_{FH} cells in total lymphocytes was calculated. A significant increase in percentage of $CXCR5^{high}PD\text{-}1^{high}$ T_{FH} cells in total lymphocytes was observed in peripheral blood from GPA patients on conventional therapies (average: 0.13) compared to age and gender matched healthy controls (average: 0.073) (P=0.037) and GPA patients following B cell depletion therapy (average: 0.077) (P=0.0044). No such difference was found between healthy controls and either B cell depleted GPA patients (P=0.66) or SLE patients (average: 0.11) (P=0.51) (Figure 4.2.5 B).

4.2.3 Frequencies of PD-1+CD4+ T cells in peripheral blood from GPA patients, healthy and disease controls

As an increased proportion of circulating T_{FH} cells was observed in GPA patients on conventional therapies but not B cell depleted GPA patients, CD4+ T cells expressing PD-1 in peripheral blood was subsequently investigated by flow cytometry. PD-1 expression was measured on CD3+CD4+ T cells in PBMC isolated from 18 GPA patients on conventional therapies, 9 B cell depleted GPA patients, 10 age and gender matched healthy controls and 8 SLE patients. The Gating strategy is shown in figure 4.2.6.

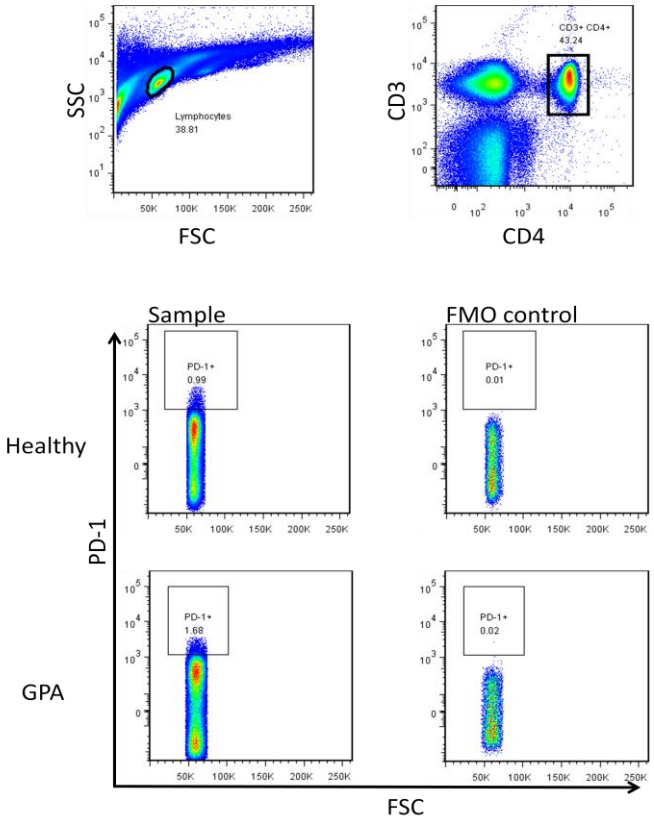

Figure 4.2.6: Gating strategy of PD-1+ cells in CD4+ T cells in peripheral blood from GPA patients on conventional therapies, B cell depleted GPA patients, healthy and disease controls. Lymphocytes identified by their forward and side scatter properties were gated for CD3+ and CD4+ double expression and then examined for expression of PD-1 according to FMO controls to identify PD-1+CD4+ T cells in peripheral blood.

A significant increase in the percentage of PD-1+ cells in CD4+ T cells was observed in peripheral blood from GPA patients on conventional therapies (average: 1.26) compared to healthy controls (average: 0.69) (P=0.0027) (Figure 4.2.7 A). However, no significant difference was found in the frequency of PD-1+ cells in CD4+ T cells between GPA patients on conventional therapies and those who had clinically responded to B cell depletion therapy (average: 0.95) (P=0.18). No difference was observed between healthy controls and either B

cell depleted GPA patients (P=0.28) or SLE patients (0.84) (P=0.83) (Figure 4.2.7 A).

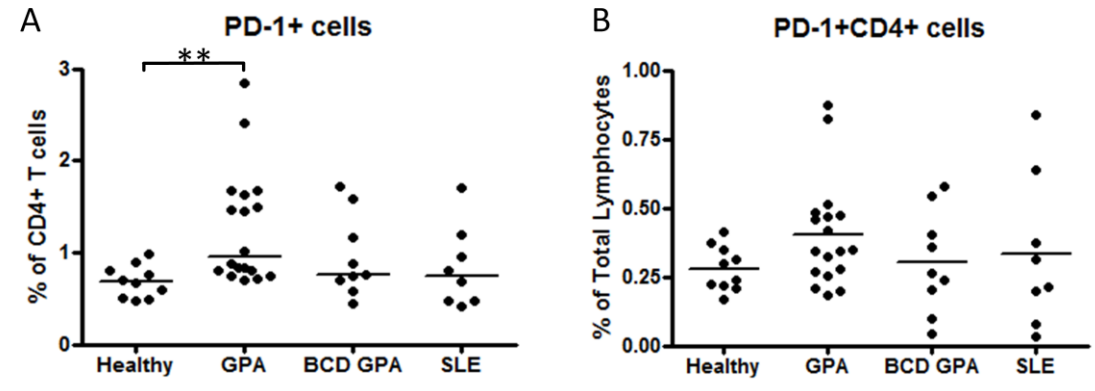

Figure 4.2.7: Frequencies of PD-1+CD4+ T cells in peripheral blood from GPA patients on conventional therapies, B cell depleted GPA patients, healthy and disease controls. **A.** Percentage of PD-1+ cells in CD4+ T cells in peripheral blood from healthy controls (average: 0.69, range: 0.47 to 0.99), GPA patients on conventional therapies (median: 0.95, range: 0.7 to 2.85) (P=0.0027 compared to healthy controls), GPA patients under B cell depletion therapy (average: 0.95, range: 0.44 to 1.72) and SLE patients (average: 0.84, range: 0.42 to 1.71). **B.** Percentage of PD-1+CD4+ T cells in total lymphocytes in peripheral blood from healthy controls (average: 0.28, range: 0.17 to 0.42), GPA patients on conventional therapies (average: 0.4, range: 0.18 to 0.87), GPA patients under B cell depletion therapy (average: 0.3, range: 0.043 to 0.58) and SLE patients (average: 0.34, range: 0.031 to 0.84).

As the proportion of CD3+ T cells in total lymphocytes varies between individuals, the frequency of PD-1+CD4+ T cells in total lymphocytes was further calculated. This time, no significant difference in percentage of PD-1 expressing CD4+ T cells in total lymphocytes was observed in GPA patients on conventional therapies (average: 0.4) compared to healthy controls (average: 0.28) (P=0.089) or B cell depleted GPA patients (average: 0.3) (P=0.32) (Figure 4.2.7 B). No difference in proportion of PD-1+ CD4+ T cells in total lymphocytes was found

between healthy controls and either GPA patients following B cell depletion therapy (P=0.84) or SLE patients (0.34) (P=0.9) (Figure 4.2.7 B).

4.2.4 Expression of PD-1 in tissues from GPA patients and controls

PD-1 positive cells were observed in 4 of the 6 biopsies from patients with GPA. As PD-1 is reported to be expressed on T_{FH} cells in germinal centres [158], tonsil sections were used as control. As expected, most PD-1 positive cells in tonsil controls were found in germinal centres (Figure 4.2.8 A). In contrast, PD-1 positive cells observed in biopsies from GPA patients tended not to be components of any organized lymphoid structure (Figure 4.2.8 B).

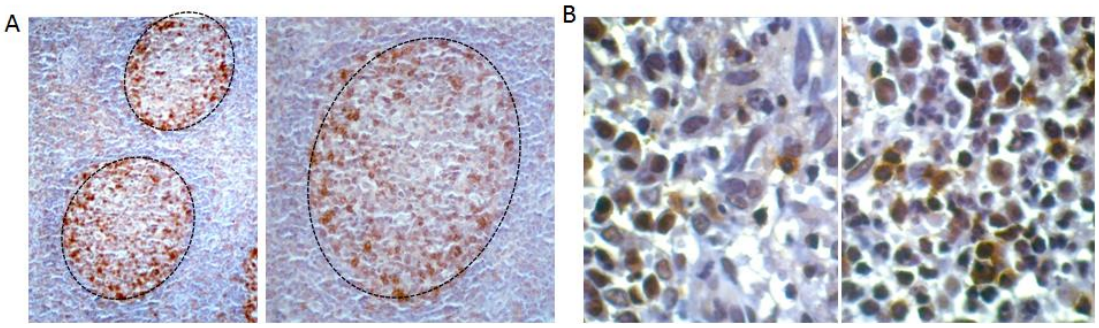

Figure 4.2.8: PD-1+ cells in paraffin sections from GPA patients were studied by immunohistochemistry. **A.** Single staining with PD-1 (brown) identified PD-1+ cells in tonsil sections, original magnifications: x100 (left), x200 (right), germinal centres in tonsil sections are indicated by circles. **B.** Single staining with PD-1 (brown) identified PD-1+ cells in biopsies from GPA patients, original magnification: x200.

4.2.5 Study of Treg cells in peripheral blood from GPA patients, healthy and disease controls

4.2.5.1 Frequencies of Treg cells in peripheral blood from GPA patients, healthy and disease controls

The frequency of Treg cells in peripheral blood from 18 GPA patients on conventional therapies, 9 B cell depleted GPA patients, 10 healthy controls and 8 SLE patients was studied by flow cytometry. Treg cells were identified in isolated PBMC as a subset of CD4+CD14- T cells that are $CD25^{high}$ CD127- (Figure 4.2.9 A). The expression of FoxP3 was examined in the Treg cell population (Figure 4.2.9 B). The majority of this population expressed FoxP3.

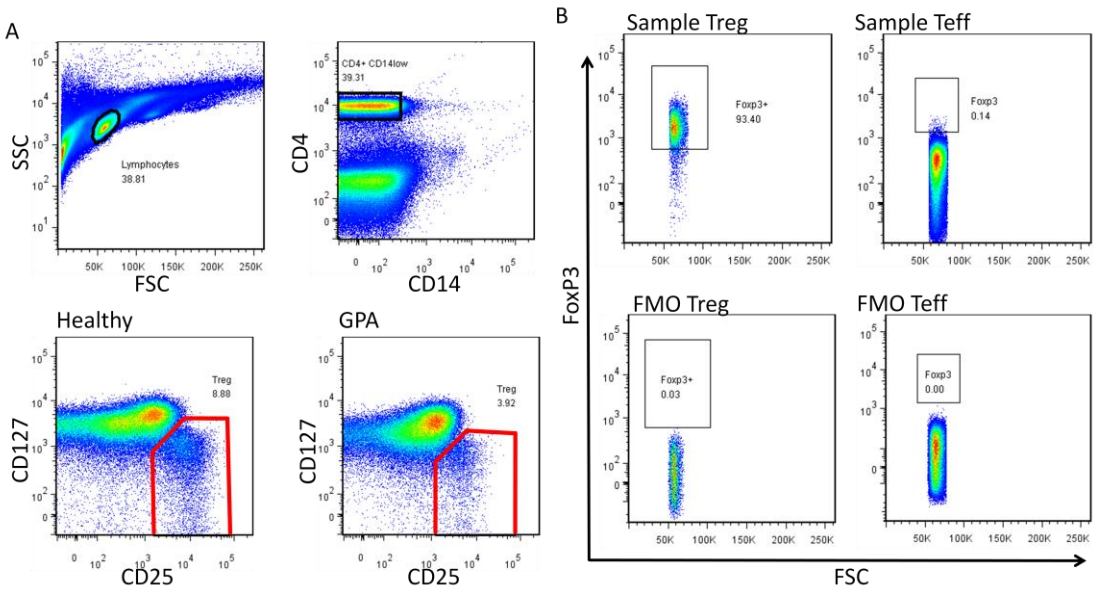

Figure 4.2.9: Gating strategy of Treg cells in peripheral blood from GPA patients on conventional therapies, B cell depleted GPA patients, healthy and disease controls. **A.** Lymphocytes identified by their forward and side scatter properties were gated for CD4+CD14- expression and then examined for expression of CD25 and CD127

to identify regulatory T cells in peripheral blood. **B.** The expression of Foxp3 in CD25+CD127- Treg cells and CD25-CD127+ T effector cells from GPA patients.

Consistent with other studies [380], Treg cells comprised on average 7.73% of CD4+ T cells in healthy controls (Figure 4.2.10 A). Interestingly, a significantly reduced proportion of Treg cells in CD4+ T cells was observed in the peripheral blood from GPA patients on conventional therapies (average: 5.03) compared to healthy controls (P=0.0017) and B cell depleted GPA patients (average: 8.04) (P=0.0043). No such difference was found between B cell depleted GPA patients and healthy controls (P=0.6). There was no difference observed in the percentage of Treg cells in CD4+ T cells in peripheral blood between healthy controls and SLE patients (average: 8.73) (Figure 4.2.10 A).

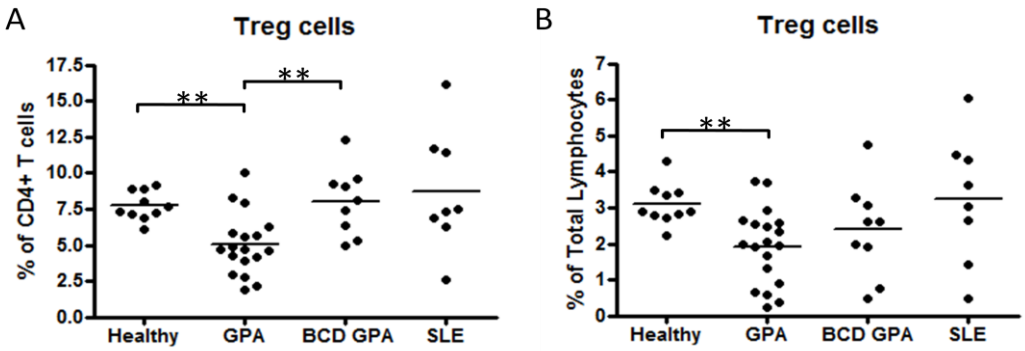

Figure 4.2.10: Frequencies of Treg cells in peripheral blood from GPA patients on conventional therapies, B cell depleted GPA patients, healthy and disease controls. **A.** Percentage of Treg cells in CD4+ T cells in peripheral blood from healthy controls (average: 7.73, range: 6.07 to 9.2), GPA patients on conventional therapies (average: 5.03, range: 1.88 to 10.07) (P=0.0017 and P=0.0043 when compared to healthy controls and GPA patients under B cell depletion therapy respectively), GPA patients under B cell depletion therapy (average: 8.04, range: 4.99 to 12.27) and SLE patients (average: 8.73, range: 2.61 to 16.16). **B.** Percentage of Treg cells in total lymphocytes in peripheral blood from healthy controls (average: 3.09, range: 2.23 to 4.29), GPA patients on

conventional therapies (average: 1.92, range: 0.22 to 3.75) (P=0.0027 when compared to healthy controls), GPA patients under B cell depletion therapy (average: 2.39, range: 0.47 to 4.76) and SLE patients (average: 3.25, range: 0.48 to 6.04).

When the frequency of Treg cells in total lymphocytes was calculated, a significant reduction was observed in GPA patients on conventional therapies (average: 1.92) compared to healthy controls (average: 3.09) (P=0.0027) (Figure 4.2.10 B). There was no difference in the proportion of Treg cells in total lymphocytes in peripheral blood between GPA patients on conventional therapies and those who clinically responded to B cell depletion therapy (average: 2.39) (P=0.3). No significant difference in Treg frequency in total lymphocytes was found between healthy controls and either B cell depleted GPA patients or SLE patients (average: 3.25) (Figure 4.2.10 B).

4.2.5.2 Frequencies of different Treg cell subsets in peripheral blood from GPA patients, healthy and disease controls

As a decrease of Treg cell frequency was observed in peripheral blood from GPA patients on conventional therapies but not B cell depleted GPA patients, different subsets of Treg cells were further studied. The Treg cell population was divided into 3 sub-populations based on the expression of CD25 and CD45RA, which represent different stages of differentiation (Figure 4.2.11 A). The reduction of Treg cell frequency in GPA patients on conventional therapies was observed in the memory and activated Treg cells but not resting Treg cells (Figure 4.2.11 B to D). The average proportion of memory Treg cells in CD4+ T

cells was 2.52 in GPA patients on conventional therapies compared to 4.72 in healthy controls and 4.63 in GPA patients following B cell depletion therapy (P=0.0001 and P=0.0022 respectively) (Figure 4.2.11 C). The average proportion of activated Treg cells in CD4+ T cells was 0.24 in GPA patients on conventional therapies compared to 0.68 in healthy controls and 0.62 in GPA patients following B cell depletion therapy (P=0.0002 and P=0.0022 respectively) (Figure 4.2.11 C).

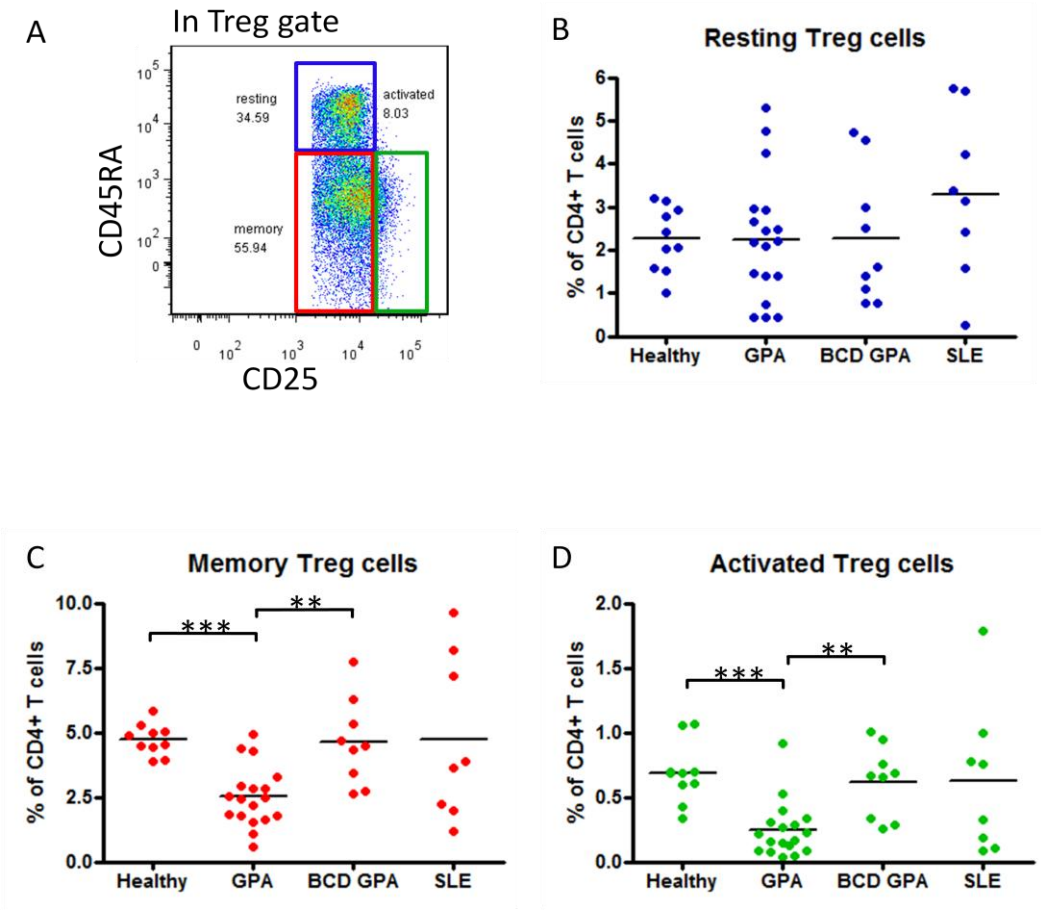

Figure 4.2.11: Frequencies of Treg cell subsets in CD4+ T cells of peripheral blood from GPA patients on conventional therapies, B cell depleted GPA patients, healthy and disease controls. **A.** Delineation of Treg subpopulations: CD25+CD127- Tregs were identified in figure 4.2.9 and then divided into resting Tregs

(CD25+CD45RA+), memory Tregs (CD25+CD45RA-) and activated Tregs ($CD25^{high}$CD45RA-). **B.** Percentage of resting Treg cells in CD4+ T cells in healthy controls (average: 2.27, range: 1 to 3.2), GPA patients on conventional therapies (average: 2.25, range: 0.44 to 5.3), GPA patients under B cell depletion therapy (average: 2.27, range: 0.76 to 4.74) and SLE patients (average: 3.3, range: 0.25 to 5.74). **C.** Percentage of memory Treg cells in CD4+ T cells in healthy controls (average: 4.72, range: 3.88 to 5.83), GPA patients on conventional therapies (average: 2.52, range: 0.6 to 4.96) (P=0.0001 and P=0.0022 when compared to healthy controls and GPA patients under B cell depletion therapy respectively), GPA patients under B cell depletion therapy (average: 4.63, range: 2.61 to 7.75) and SLE patients (average: 4.74, range: 1.18 to 9.63). **D.** Percentage of activated Treg cells in CD4+ T cells in healthy controls (average: 0.68, range: 0.34 to 1.07), GPA patients on conventional therapies (median: 0.20, range: 0.04 to 0.91) (P=0.0002 and P=0.0022 when compared to healthy controls and GPA patients under B cell depletion therapy respectively), GPA patients under B cell depletion therapy (average: 0.62, range: 0.25 to 1), and SLE patients (average: 0.63, range: 0.088 to 1.78).

As the frequencies of Treg cells and CD4+ T cells in total lymphocytes differ between individuals, the proportion of each Treg subset in total lymphocytes was further studied. No difference was observed in the frequency of resting Treg cells in total lymphocytes between GPA patients, healthy controls and SLE patients (Figure 4.2.12 A). The frequency of memory Treg cells in total lymphocytes was significantly lower in GPA patients on conventional therapies (average: 0.87) compared to healthy controls (average: 1.88) (P<0.0001) and B cell depleted GPA patients (average: 1.41) (P=0.033). No such difference was observed between B cell depleted GPA patients and healthy controls (Figure 4.2.12 B). The frequency of activated Treg cells in total lymphocytes was significantly lower in GPA patients on conventional therapies (average: 0.088) compared to healthy controls (average: 0.27) (P<0.0001) and B cell depleted

GPA patients (average: 0.18) (P=0.0081). No such difference was observed between B cell depleted GPA patients and healthy controls (Figure 4.2.12 C). No difference in any Treg subset was found between SLE patients and healthy controls.

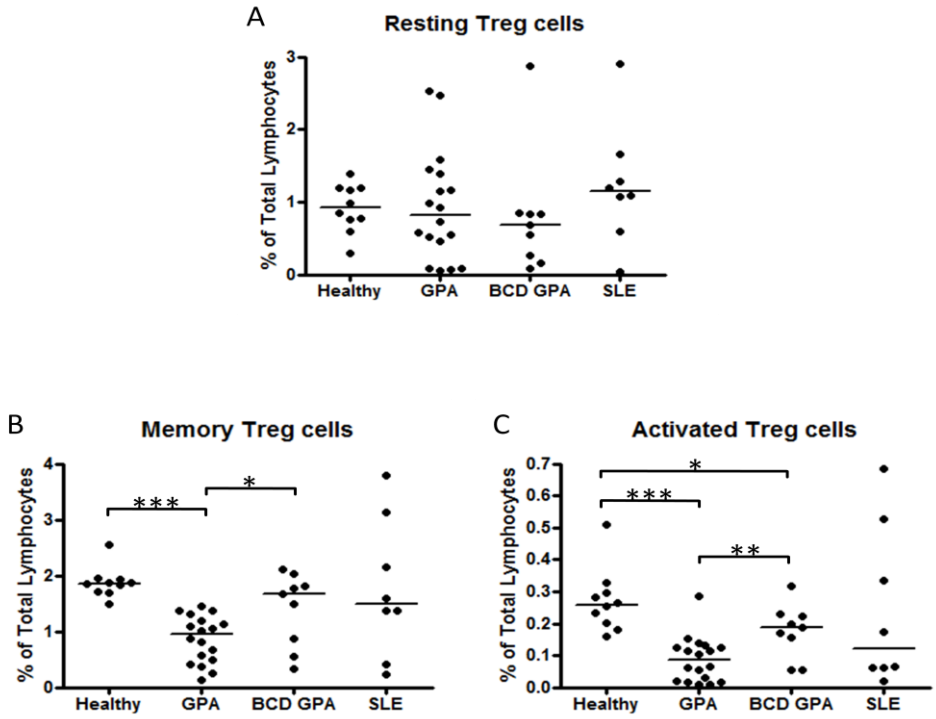

Figure 4.2.12: Frequencies of Treg cell subsets in total lymphocytes of peripheral blood from GPA patients on conventional therapies, B cell depleted GPA patients, healthy and disease controls. **A.** Percentage of resting Treg cells in total lymphocytes in healthy controls (average: 0.92, range: 0.29 to 1.39), GPA patients on conventional therapies (average: 0.93, range: 0.059 to 2.54), GPA patients under B cell depletion therapy (median: 0.69, range: 0.081 to 2.88) and SLE patients (average: 1.23, range: 0.047 to 2.91). **B.** Percentage of memory Treg cells in total lymphocytes in healthy controls (median: 1.86, range: 1.5 to 2.56), GPA patients on conventional therapies (average: 0.87, range: 0.13 to 1.45) (P<0.0001 and P=0.033 when compared to healthy controls and GPA patients under B cell depletion therapy respectively), GPA patients under B cell depletion therapy (average: 1.41, range: 0.33 to 2.11) and SLE patients (average: 1.76, range: 0.23 to 3.79). **C.** Percentage of activated Treg cells in total lymphocytes in healthy controls (median: 0.26, range: 0.16 to 0.51), GPA patients on conventional therapies (median: 0.086, range: 0.00079 to 0.28) (P<0.0001 and P=0.0081

when compared to healthy controls and GPA patients under B cell depletion therapy respectively), GPA patients under B cell depletion therapy (average: 0.18, range: 0.053 to 0.32), and SLE patients (average: 0.24, range: 0.02 to 0.68).

4.2.5.3 Functional study of Treg cells from GPA patients and healthy controls

Suppressive functions of Treg cells from 3 GPA patients on conventional therapies and 3 healthy controls were investigated. Clinical information of patients studied for Treg cell function is shown in table 4.2.4.

Table 4.2.4: Clinical information of GPA patients studied for Treg cell function

		BVAS	Antibody	Gender	Age	Disease duration (yrs)	Organ involvement	Current treatment
GPA	P-1	6	neg	M	51	9	sinuses, orbit, lungs, CNS	completed CYC, prednisolone, adcal D3, risedronate, omeprazole, carbamazepine
	P-2	3	ANCA	F	58	>2	sinuses	Azathioprine, cetrizine, septrin, calcichew, vit D, lansoprazole, quinine, ramipril, simvastatin, alendronic acid, amlodipine
	P-3	6	ANCA	M	64	11	lungs, renal	MTX, folic acid. Pred, adcal D3, warfarin, omeprazole, rosuvastatin, ramipril

As different concentration of stimuli can cause different suppression rate, 3 different concentrations of anti-CD3/CD28 stimuli were used in the functional study. Consistent with the observation in section 4.2.5.1, a significant reduction in Treg cell frequency in peripheral blood from GPA patients on conventional therapies (average: 3.3) (P=0. 0087) was detected compared to 3 matched healthy controls (average: 6.9) (Figure 4.2.13 A). Treg cells isolated from both GPA patients and matched healthy controls were observed to suppress T effector cell proliferation, regardless of the concentration of stimuli (Figure 4.2.13 C and D). No difference in Treg cell-mediated suppression of T effector cell

proliferation was found between GPA patients on conventional therapies and healthy controls (Figure 4.2.13 B, P=0.23).

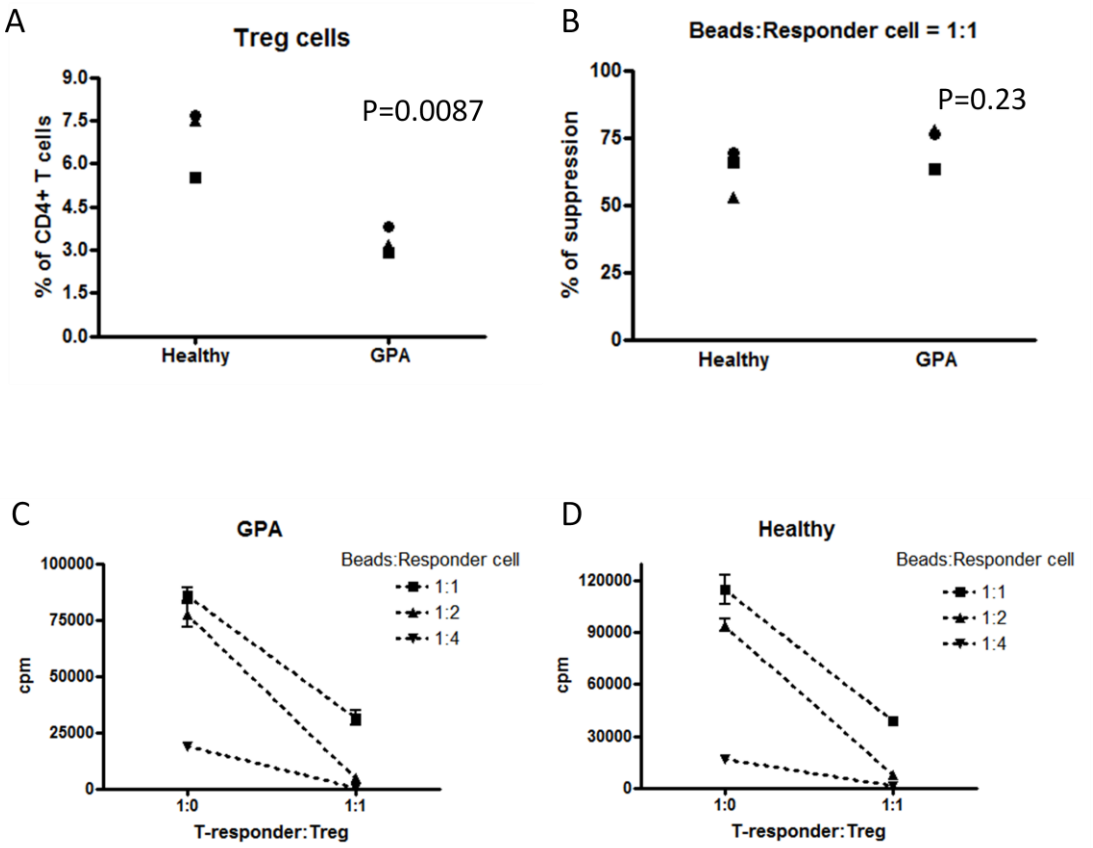

Figure 4.2.13: Functional study of Treg cells from GPA patients and healthy controls. **A.** Percentage of CD25+CD127- Treg cells in CD4+ T cells in 3 GPA patients (median: 3.2, range: 2.9 to 3.8) and 3 healthy controls (median: 7.5, range: 5.5 to 7.7) (P=0.0087). **B.** Suppression rate of Treg cells from 3 GPA patients (median: 76.38, range: 63.24 to 77.94) and 3 paired healthy controls (median: 66.05, range: 52.8 to 69.22) activated at a bead:conventional-cell ratio of 1:1 (P=0.23). **A and B:** The 3 pairs of GPA patients and healthy controls were represented with circle, square and triangle. **C.** Radioactivity measurement of T effector cells alone or co-cultured with Treg cells (1:1) from 1 GPA patient activated at a bead: conventional-cell ratio of 1:1, 1:2 or 1:4. **D.** Radioactivity measurement of T effector cells alone or co-cultured with Treg cells (1:1) from 1 healthy control activated at a bead: conventional-cell ratio of 1:1, 1:2 or 1:4.

4.2.6 Study of FoxP3+ Treg cells in tissues from GPA patients and controls

To study Treg cells in lesions from GPA patients, expression of FoxP3 and CD3 on biopsies from GPA patients and tonsil controls was studied. CD3+ T cells were observed in 6 of the 7 biopsy sections from GPA patients and controls. The numbers of CD3+ T cells infiltrating in the biopsies were variable between individuals. FoxP3+ Treg cells were observed in all sections that contained CD3+ T cells (Figure 4.2.14). The proportions of FoxP3+ Treg cells in CD3+ T cells in each section were variable.

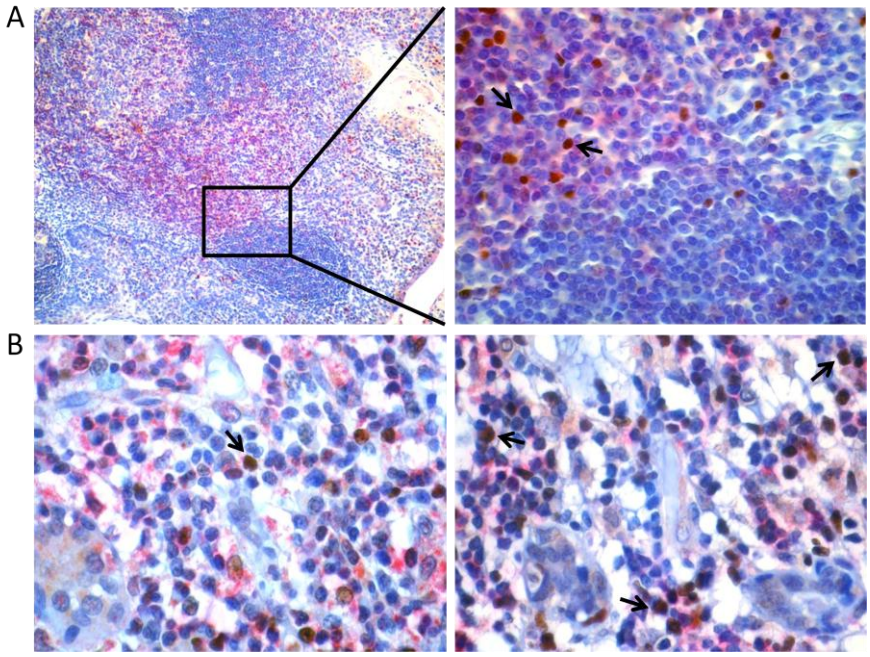

Figure 4.2.14: Foxp3+CD3+ T cells in paraffin sections from GPA patients were studied by immunohistochemistry. **A.** Double staining with FoxP3 (brown) and CD3 (pink) identified Treg cells in tonsil sections, original magnifications: x100 (left), x400 (right). **B.** Double staining with FoxP3 (brown) and CD3 (pink) identified Treg cells in biopsies from GPA patients, original magnification: x400.

4.2.7 Correlation between T_{FH} and Treg cell frequencies in human peripheral blood

A significantly increased frequency of circulating T_{FH} cells and a significant reduction in Treg cell frequency was observed in peripheral blood from GPA patients on conventional therapies compared to healthy controls and B cell depleted GPA patients in previous sections. Therefore a numerical relationship between frequencies of circulating T_{FH} cells and Treg cells in human blood was hypothesised. Proportions of circulating T_{FH} cells and Treg cells in CD4+ T cells in peripheral blood from all GPA patients, healthy controls and SLE patients were plotted on an XY graph. A significant inverse correlation between the frequencies of circulating T_{FH} cells and Treg cells in CD4+ T cells was observed (Figure 4.2.15 A, P=0.012).

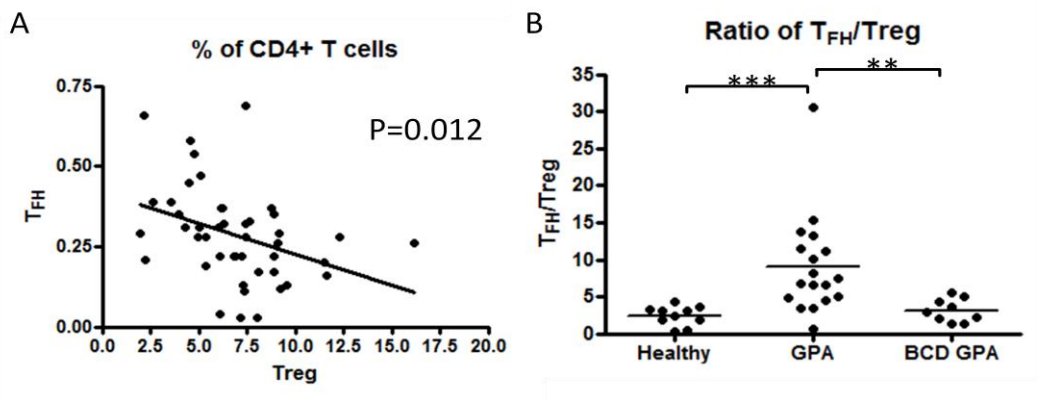

Figure 4.2.15: Analysis of frequencies of T_{FH} cells and Treg cells in CD4+ T cells in peripheral blood. **A.** Circulating T_{FH} cell frequency and Treg cell frequency from all samples studied in this thesis, including GPA patients, BCD GPA patients, SLE patients and healthy controls, was significantly inverse correlated in CD4+ T cells (P=0.012, r^2=0.14). **B.** Ratio of circulating T_{FH} versus Treg frequencies in CD4+ T cells from 18 GPA patients (average: 9.07, range: 0.71 to 30.56) and 10 healthy controls (average: 2.43,

range: 0.37 to 4.33) (P= 0.0002); and 9 BCD GPA patients (average: 3.16, range: 1.3 to 5.61) (P=0.0031).

Since a mathematical relationship between circulating T_{FH} cells and Treg cells was observed, the ratio of these two populations was hypothesised to provide better discrimination between GPA patients and healthy controls than circulating T_{FH} or Treg frequencies alone. Therefore the ratio of circulating T_{FH} cells to Treg cells in peripheral blood from GPA patients on conventional therapies, GPA patients following B cell depletion therapy and healthy controls was further investigated. A clearer discrimination between GPA patients on conventional therapies (average: 9.07) and healthy controls (average: 2.43) (P=0.0002) and GPA patients following B cell depletion therapy (average: 3.16) (P=0.0031) was observed. No difference in the ratio of circulating T_{FH} cells versus Treg cells was found between GPA patients following B cell depletion therapy and healthy controls (Figure 4.2.15 B, P=0.5).

4.2.8 Main findings in this chapter:

- Higher frequency of circulating $CXCR5^{high}PD\text{-}1^{high}$ T_{FH} cells in peripheral blood was observed from GPA patients on conventional therapies compared to healthy controls and GPA patients following B cell depletion therapy.
- Higher proportion of PD-1 expressing CD4+ T cells was observed in peripheral blood from GPA patients on conventional therapies compared to healthy controls.
- Lower frequency of Treg cells in peripheral blood was observed from GPA patients on conventional therapies compared to healthy controls and GPA patients following B cell depletion therapy.
- No difference in the the capacity of Treg cells to suppress T cell proliferation was found between GPA patients on conventional therapies and healthy controls.
- The frequencies of circulating T_{FH} and Treg cells in CD4+ T cells were found to be inversely correlated in human peripheral blood.

4.3 Discussion

4.3.1 B cell depletion therapy in GPA

GPA is a condition where the pathogenicity of autoantibodies may drive the vasculitis and renal pathology directly. Consistent with previous studies [381], cANCA titers from 4 out of 9 patients in our study did not change after B cell depletion therapy despite a good clinical response (Table 4.2.2), suggesting that B cell depletion alone is unlikely to be the sole basis for the success of rituximab in generating favorable clinical outcomes in GPA. The frequencies of regulatory T cell subsets are also likely to impact on disease activity.

4.3.2 Circulating T_{FH} cells in GPA

The frequency of circulating T_{FH} cells in peripheral blood from GPA patients on conventional therapies and GPA patients following B cell depletion therapies has been described for the first time in this thesis. An increase in circulating T_{FH} cell frequencies was observed in peripheral blood from GPA patients on conventional therapies. This finding is consistent with previous studies on patients with severe SLE [158].

T_{FH} cells are cells expressing high level of CXCR5, located in germinal centres that support affinity maturation and the production of high affinity Abs [153, 382, 383]. The pathway of T_{FH} cell differentiation is not clear yet. Some studies

indicated that T_{FH} cells are a distinct population developed from activated CD4+ T cells in B cell dependent or independent ways [148, 384]. Others indicated that T_{FH} cells could develop from Th2 cells [149], while a recent study reported that T_{FH} cells can develop into other helper T cells in mice [385]. Despite this disagreement, T_{FH} cells have been demonstrated to be involved in autoimmune diseases in studies on both animal models and patients. Studies of mouse models have shown that T_{FH} cells are required for germinal centre formation and play an important role in generation of systemic autoimmunity from a germinal centre microenvironment [222]. Germinal centres have been reported in many animal models of autoimmune diseases [386]. T_{FH} like cells have been observed in the spleen in mouse models of lupus [387, 388]. In patients with SLE, auto-reactive B cells have been observed within germinal centres [389]. Although there is a large population of CXCR5+CD4+ T cells found in human peripheral blood, they may not be the same population as T_{FH} cells in germinal centres [136, 390]. Further studies indicated that in addition to high expression of CXCR5, co-expression of high levels of ICOS or PD-1 could be used to identify circulating T_{FH} cells in human peripheral blood [150]. One study of patients with SLE has observed an expansion of $CXCR5^{high}PD\text{-}1^{high}$ circulating T_{FH} cells in peripheral blood from a subset of patients with the most severe presentation of SLE [158]. Consistent with previous studies of SLE, a higher frequency of circulating T_{FH} cells has been observed in blood of GPA patients on conventional therapies contains compared to controls. Interestingly, there was

statistically no difference observed in the frequency of circulating T_{FH} between the group of GPA patients who had responded to B cell depletion therapy and the healthy control group. Since T_{FH} are physiologically associated with B cell activation, survival and selection for specificity during the germinal centre response, an increase in circulating T_{FH} in active disease could reduce the threshold for B cells to produce pathogenic Abs in GPA.

4.3.3 PD-1+CD4+ T cells in GPA

In this thesis, a significantly increased proportion of PD-1+ CD4+ T cells was observed in GPA patients (average: 1.26, range: 0.7 to 2.85) compared to healthy controls (average: 0.69, range: 0.47 to 0.99). Since the experiments for this study were completed, a manuscript reporting an increased proportion of PD-1 expressing CD4+ T cells (average 9.8, range: 6.7 to 17.7), in particular antigen experienced CD4+ T cells, in peripheral blood from GPA patients was published [391]. In contrast to the published paper, much lower frequencies of PD-1+ cells were identified in this thesis, although the trend was the same. Differences between the data might due to different staining and gating methods used for different patient cohorts. A reduction in functional suppression mediated by PD-1 in GPA patients was also reported by this study, although the possible suppressive effect of Treg cells and other immune suppressors was not excluded in the experimental protocol [391]. Fewer activated and memory

Treg cells in GPA on conventional therapies compared to healthy controls has been described in this thesis. The suppressive capacity per cell in Treg cells in GPA was shown to be the same as in healthy controls in this thesis. Therefore the suppression of immune responses in the study of Wilde *et al* 2012 [391], which the authors suggested impaired PD-1 mediated immune suppression in GPA, could have been influenced by altered frequency of Treg cells that have been identified in this thesis in GPA patients.

4.3.4 PD-1+ cells in lesions of GPA

Cells expressing PD-1 in biopsies from GPA patients and controls have been studied in this thesis. Consistent with previous studies, PD-1+ cells were observed in germinal centres of tonsil controls [392]. In contrast, PD-1+ cells were observed without lymphoid tissue structure in biopsies from GPA patients.

Expression of PD-1 in human tissues has been described and is associated with autoimmune diseases. PD-1 has been observed to be expressed by T_{FH} cells in germinal centres, T cells in T cell zones and B cells follicles in human tonsil [392]. PD-1 expressed by CD4+ T cells and B cells in livers from patients with autoimmune liver diseases was observed. Higher relative quantity of mRNA encoding PD-1 was detected in livers with autoimmune liver disease compared to normal controls [296]. Increased PD-1 expression by CD4+ T cells and CD8+

T cells in salivary gland from patients with Sjögren's Syndrome was detected [393]. Since the experiments for this study were completed, a manuscript reporting a lack of PD-1 expression on T cells in renal biopsies from GPA patients has been published [391]. In contrast to their study, few PD-1+ cells were detected in biopsies from GPA patients in this thesis. Difference in the data might be due to different organs that were biopsied and different patient cohorts. Germinal centre like structures have been described in biopsies from GPA patients, although B cells in most samples of mucosa involved in GPA patients are distributed diffusely (as described in chapter 3) [326, 394, 395]. However, PD-1+ cells detected in biopsies from GPA patients were relatively infrequent and not specifically associated with either the B cell infiltrate or organized lymphoid structure. Due to the small size of biopsies and limited numbers of lymphocyte infiltrating, it is difficult to determine whether these PD-1+ cells generally contribute to local inflammation in GPA patients. Moreover, as it is very difficult to obtain biopsies or lymph nodes from GPA patients when they are following B cell depletion therapy, it is difficult to see how B cell depletion could affect mucosal cells with the T_{FH} cells in GPA.

PD-1 has been shown to be expressed on lymphocytes in different organs in mouse models. In contrast to young mice, PD-1 was found to be expressed by un-stimulated CD4+ T cells in spleen of aged mice. Furthermore, more PD-1+ CD4+ T cells and higher expression level of PD-1 on CD4+ T cells in spleen was

observed from old mice compared with young mice [396]. This is confirmed by a study of a mouse model of lupus-like nephritis where PD-1 expressing CD4+ T cells were also observed infiltrating the kidney [397]. Another study reported that PD-1 is expressed by CD3+ T cells in spleen, thymocytes, and B cells in germinal centres from draining lymph nodes in mice. Additionally PD-1 was reported to be up-regulated on infiltrating mononuclear cells from pancreas in NOD mice and brain in experimental autoimmune encephalomyelitis mice [302]. Therefore it would be interesting to study PD-1 expression in more biopsies from different organs of GPA patients to see if such variation exists. Expression of PD-1 and its ligands in GPA was considered to be interesting and possibly related to disease pathogenesis and will be studied in more detail in chapter 5.

4.3.5 Treg cells in GPA

The frequency of Treg cells in peripheral blood from GPA patients has been studied in this thesis. A reduction in Treg cell frequency in peripheral blood from GPA patients on conventional therapy but not GPA patients following B cell depletion therapy was observed compared to healthy controls.

Previous studies have shown that reduction in frequency of Treg cells or impaired suppressive capacity of Treg cells may result in failure to suppress immune responses, which may predispose to autoimmunity [398-400]. Some

studies have observed a decreased proportion of Treg cells in GPA [338], while others found an increase or no difference in percentages of Treg cells between GPA patients and healthy controls [336, 337]. The differences might due to different gating strategies and different patient cohorts and therapies [1, 2, 401]. Consistent with the findings of Rimbert *et al* [338], a significantly reduced frequency of Treg cells was observed in GPA patients on conventional therapies compared to healthy controls. Interestingly, this reduction was only found in the memory and activated Treg populations, which are generated *in vivo* as a consequence of antigen driven activation and are the Treg populations responsible for immune resolution [180, 402].

One study has reported a decreased proportion of Treg cells in peripheral blood but an increase of Treg cell proportion in gut from patients with Crohn's disease, suggesting that the reduction of Treg cells in peripheral blood may due to migration to inflamed tissue [403]. Although FoxP3+CD3+ Treg cells were observed in 6 out of 7 biopsies from GPA patients in this chapter, the total number of CD3+ T infiltrating in biopsies was variable and the percentage of FoxP3+ Treg cells in CD3+ T cells was quite different between biopsies. In contrast to biopsies from gut, most biopsies studied in this thesis were from upper airways, eyes and bronchi of GPA patients. Due to the small size of biopsies and the small number of infiltrating CD3+ T cells, it is hard to conclude

whether the reduction of circulating Treg cells in GPA patients could be due to migration to inflamed tissue, though it would seem unlikely.

Previous studies have reported a normal or increased percentage of Treg cells in peripheral blood from GPA patients but with an impaired suppressive efficiency compared to healthy controls [336, 337]. In this chapter, the capacity of Treg cells to suppress T cell proliferation in 3 GPA patients, two of whom were cANCA and PR3 positive, while one was ANCA negative, were studied. In contrast to previous studies, the suppressive efficiency of Treg cells in 3 cases of GPA in this study was equivalent to that of healthy controls regardless of the ANCA status of the GPA patients. This may be due to differences in sorting strategy when isolating Treg cells and effector T cells. Sorting strategy used in this thesis had higher stringency compared to other studies [336, 337].

4.3.6 Correlation between circulating T_{FH} cells, Treg cells and B cells

The relative frequencies of circulating T_{FH} cells and Treg cells in peripheral blood from GPA patients following B cell depletion therapy and the inverse correlation between these two populations in human has been described for the first time in this thesis.

Since the experiments for this study were completed, two studies from a US lab reporting expansion of circulating T_{FH} cells in peripheral splenocytes from Treg

cell deficient mice have been published [404, 405]. The deficiency of Treg cells and expansion of circulating T_{FH} cells in those mice was demonstrated to result in a loss of B cell anergy [404, 405]. Consistent with their findings, the frequencies of circulating T_{FH} cells and Treg in peripheral blood in this thesis were found to be inversely related and potentially dependent on B cells. Therefore, Treg cell frequencies in peripheral blood may depend on B cells through a link with circulating T_{FH} cells which have the same characteristics as T_{FH} cells in germinal centres. Germinal centre structures have been reported to be lost in patients following B cell depletion treatment [406]. This implies the expansion of circulating T_{FH} cells and reduction of Treg cells in peripheral blood from GPA patients may contribute to the disease pathogenesis.

Previous studies have indicated a relationship between B cell deficiency and T cell activation in an animal model of SLE. T cell activation was shown to be inhibited in B cell deficient mice [407], indicating a potential effect of B cell depletion on T cell responses. Further studies demonstrated that SLE patients following B cell depletion therapy have augmented Foxp3 mRNA and more Treg cells compared to patients who were not in remission [339, 408, 409]. Restoration of Treg cell numbers by B cell depletion has been also observed in animal models of arthritis [410]. In this chapter, a decreased frequency of Treg cells in peripheral blood from GPA patients on conventional therapies was observed compared to healthy controls, while this difference was not detected in

GPA patients following B cell depletion therapy implying that B cell depletion restores Treg numbers. However, whether the nature of Treg cell restoration to normal levels in peripheral blood observed following B cell depletion is a cause or consequence of disease resolution is unclear.

The ratio between circulating T_{FH} cell and Treg cell numbers could be used as a means of heightening the sensitivity of a test to detect differences between GPA patients and controls or predict clinical outcomes. Despite the sensitivity of the ratio, no difference between GPA patients following B cell depletion therapy and healthy controls was observed. This supports the concept that functional T cell equilibrium could be restored by B cell depletion therapy, and therefore implies that these T cell subsets could be possibly B cell dependent, though at the moment cause and effect are impossible to separate.

The identification of two regulatory T cell subsets that are inversely related in frequency and potentially B cell dependent implies that there may be a developmental relationship between them or development of these two cell types may be altered during T cell differentiation. Tsuji *et al* has suggested that T_{FH} cells and Treg cells are linked developmentally in the gut in a B cell dependent way in a mouse model [411]. However, other studies in animal models have reported that PD-1 ligand PD-L1 could negatively regulate the generation of T_{FH} cells while positively regulate the development of Treg cell

[166, 282]. PD-1 and its ligands were therefore further studied in the next chapter.

4.3.7 Conclusion

- Higher frequency of circulating T_{FH} cells and PD-1+ T cells observed in peripheral blood from GPA on conventional therapies may contribute to the pathogenesis of GPA
- Although the capacity of Treg cells to suppress T cell proliferation in GPA patients was found to be the same as in healthy controls, the lower frequency of Treg cells found in peripheral blood from GPA patients on conventional therapies may lead to less suppression and contribute to autoimmune responses in GPA.
- The frequencies of circulating T_{FH} cells and Treg cells in peripheral blood from GPA patients could be altered by B cell depletion therapy which implies these two populations are B cell dependent.
- It may be possible to consider the ratio between the inversely correlated frequencies of circulating T_{FH} cells and Treg cells in peripheral blood as a biomarker for disease relapse in GPA.

Chapter 5

Study of PD-1 and its ligands in GPA

5.1 Introduction

The immunoglobulin superfamily member PD-1 is expressed by circulating T_{FH} cells and therefore antibody to PD-1 was used as a component of a panel of Abs to identify circulating T_{FH} cells in peripheral blood in the previous chapter [158]. However PD-1 and its B7 family ligands PD-L1 and PD-L2 [246, 291] are of interest in their own right in the context of the pathogenesis of autoimmune diseases [412], and have therefore been studied in more detail in this chapter.

An association between abnormal PD-1 function and human autoimmune disease has been identified by genomic studies. A susceptibility locus for SLE was identified on chromosome 2q37 by a genomic screen [255, 256]. Subsequently, the *pdcd1* gene encoding PD-1 was located within the region 2q37.3 and was found to associate with susceptibility in SLE with a relative risk of up to 3.5 [257]. Various studies illustrated that the polymorphisms in *pdcd1* were associated with susceptibility to various autoimmune diseases, including rheumatoid arthritis, type 1 diabetes and ankylosing spondylitis, from different ethnic groups, as well as SLE [259, 260, 262-266]. Polymorphisms in the *CD274* gene which encodes PD-L1 were found to be associated with Graves' disease in Asian and Caucasian cohorts [285, 286], and with ankylosing spondylitis in Chinese patients [266, 287]. A polymorphism in *pdcd1lg2* which encodes PD-L2 was found to be associated with SLE and ankylosing spondylitis in Chinese cohorts [287, 289]. Polymorphisms in the *pdcd1*, *CD274* and *pdcd1lg2* genes have not

been thoroughly studied in GPA. One paper reported that polymorphisms in *pdcd1* were not associated with GPA [340]. No disease association with polymorphisms in PD-L1 or PD-L2 have been reported in GPA.

The importance of PD-1 in the pathogenesis of autoimmunity has been suggested by mouse models. Deficiency of PD-1 in several mouse models resulted in the development of lupus-like autoimmune disease or accelerated the onset of type 1 diabetes [413, 414]. Some studies indicated that expression of PD-1 alone does not trigger cell death, and emphasized the importance of interactions between PD-1 and its ligands PD-L1 or PD-L2 [239]. Blockade of the PD-1/PD-L1 pathway promoted T effector cell proliferation and accelerated type 1 diabetes in NOD mice [415, 416]. PD-1/PD-L1 interaction was shown to down-regulate immune responses by both inhibiting naïve T cell differentiation into T effector cells and also affecting cytokine production [236]. Further study of mouse models illustrated that PD-1/PD-L1 interaction down-regulated the expansion of T_{FH} cells but up-regulated the development of Treg cells [166, 282]. Moreover, PD-L1 is also thought to associate with Treg function as one animal study indicated that PD-L1 deficient Treg cells were defective in inhibiting Th1 responses in mice [283]. Recently, Treg cells were shown to suppress autoreactive B cells through a PD-1/PD-L dependent pathway [284].

As described above, PD-1 and its ligands PD-L1 and PD-L2 are implicated in autoimmune pathogenesis and have immune-regulatory functions. Expression

of PD-1 and its two ligands PD-L1 and PD-L2 were investigated in GPA in this chapter.

VISTA is a novel member of the Ig superfamily, which is structurally similar to PD-L1 though it does not bind PD-1. VISTA has been shown to be involved in suppressing T cell-mediated immune responses in an autoimmune disease model [311]. VISTA was therefore also investigated to see whether there is any difference in the level of VISTA mRNA transcripts in different cell types between patient groups and healthy controls.

Aims of this chapter:

PD-1, its ligands PD-L1 and PD-L2, and VISTA are immune-regulatory molecules associated with development of autoimmunity. The aim of this chapter was to identify any differences in the expression of PD-1, PD-L1, PD-L2, and VISTA in PBMC from GPA patients, healthy and disease controls.

5.2 Results

Some of the data studied in this chapter are not normally distributed, therefore nonparametric statistics including the Mann-Whitney U- test were used to analyse data in this chapter. Clinical information of patients studied in this chapter is shown in table 5.2.1-5.2.3.

Table 5.2.1 Clinical information of GPA patients on conventional therapies

		Gender	Age	Disease duration (yrs)	Ab	Ab titers		BVAS	Organ involvement	ISD	
						PR3	MPO			Previous	Sample date
GPA	P1	M	67	5	CANCA	17.5	0.9	7	lungs skin ears nose eyes cns sinus	MTX, CYC, Pred	MTX, Pred
	P2	F	33	2	cANCA	37.2	0	14	lungs, sinus, renal, skin, nerves	MTX, CYC, Pred, AZA	AZA, Pred
	P3	M	51	3	cANCA	36.5	0	4	sinuses, renal, skin, lungs	MTX, Pred	MTX, Pred
	P4	M	48	8	cANCA	>600	0	2	renal	MMF, Pred	MMF, Pred
	P5	F	64	2	cANCA	23.5	0	31	sinus ears nose lungs skin eyes cns	CYC, PEX, AZA, Pred	AZA, Pred
	P6	F	45	10	cANCA	71.2	0	20	ears, nose, sinus, nerves, joints, lungs	CYC, MMF, Pred	MMF, Pred
	P7	M	25	<1	cANCA	212	85	30	sinuses, orbit, renal, nerves, trachea, lungs	CYC, Pred	AZA, Pred
	P8	M	60	13	cANCA	38.2	1.4	2	sinus, renal, skin	AZA, Pred, CYC	AZA, Pred
	P9	M	44	5	cANCA	14	0	2	sinus, skin, joints	MTX, Pred	Pred
	P10	F	55	3~4	cANCA	0.4	3.4	13	renal, ears, nose, trachea, nerves	MMF, AZA, Pred	MTX, Pred

Table 5.2.2 Clinical information of GPA patients following BCD treatment

		Gender	Age	Disease duration (yrs)	Ab	ANCA titers				BVAS		Organ involvement	Current ISD
						pre-BCD		after-BCD		pre-BCD	after-BCD		
						PR3	MPO	PR3	MPO				
BCD GPA	P1	M	55	>20	cANCA	3.6	0.9	2.9	0.9	18	5	sinuses, orbit	MTX, Pred
	P2	M	63	6	cANCA	264	0.7	10.3	0	31	3	sinuses, renal,	AZA, Pred
	P3	M	55	>20	cANCA	192	0.2	8.8	0	17	2	sinuses, eyes, ears, nerves, lungs	Pred
	P4	M	54	20	neg	0	0	0	0	10	3	sinuses, ears	AZA, Pred
	P5	F	73	<1	cANCA	31.4	3.4	0.9	0	35	7	sinuses, ears, renal, lungs, nerves, skin	AZA, Pred
	P6	M	68	10	neg	0	0	0	0	4	4	sinuses, nerves, autoimmune bone marrow suppression	Pred
	P8	F	42	4	cANCA	8.1	0.6	8.1	0	17	13	sinuses, ears, orbit, trachea, lungs, brain	Pred

Table 5.2.3 Clinical information of SLE patients

		Gender	Age	Antibody	Disease duration (yrs)	BILAG SCORES									Total BILAG score	Organ involvement	Current treatment
						Constitutional	Mucocutaneous	Neuropsychiatric	Musculoskeletal	Cardiorespiratory	Gastrointestinal	Ophthalmic	Renal	Haematological			
SLE	P1	F	37	ANA dsDNA ENA Ro La	14	D	D	E	D	D	E	E	E	D	0	lupus rash arthritis in remission	HCQ Pred
	P2	F	36	ANA dsDNA ENA Ro Sm RNP	14	A	B	A	B	B	E	E	E	D	48	lupus rash cerebral lupus serositis arthritis	MMF HCQ Pred
	P3	F	50	ANA dsDNA ENA Ro Sm ACP	7	C	D	E	C	E	E	E	D	E	2	lupus nephritis II+V cerebral lupus arthritis in remission	AZA HCQ Pred
	P4	F	66	ENA Ro	20	C	C	E	C	E	E	E	E	E	3	arthritis lupus rash in remission	HCQ Pred
	P5	F	22	ANA dsDNA	3	D	D	D	C	E	E	E	E	C	2	lupus rash arthritis cns serositis in remission	HCQ Pred
	P6	F	41	ANA	22	D	D	D	C	E	E	E	D	D	1	lupus nephritis V in remission	MTX Pred
	P7	F	41	ANA dsDNA ena rnp	9	B	B	E	C	C	E	E	E	C	19	lupus rash serositis arthritis vasculitis	MTX Pred
	P8	F	47	ANA	15	D	D	E	D	E	E	E	E	E	0	lupus rash arthritis alopecia in remission	HCQ
	P9	F	53	ANA dsDNA LAC	32	D	D	E	D	E	E	E	E	E	0	lupus rash arthritis in remission	Mepacrine

5.2.1 Quantification of PD-1, PD-L1 and PD-L2 expression by PBMC in GPA patients, healthy and disease controls

The relative quantity of mRNA encoding PD-1 and its ligands PD-L1 and PD-L2 was initially quantified in PBMC by RT-PCR. cDNA was prepared from RNA isolated from PBMC of 15 GPA patients on conventional therapies, 13 age and gender matched healthy controls, 8 GPA patients under B cell depletion therapy and 15 SLE patients. The method was optimized before application to cDNA from all samples. Primers for all targets were checked with a sorted CD4+ T cell sample at two dilutions to ensure that approximately the same relative quantity value was obtained after correction for dilutions (Table 5.2.4). All RT-PCR data in this chapter is expressed as delta CT, where expression of a target gene is standardised according to expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), so that expression of GAPDH would be 1.

Table 5.2.4: Optimisation of RT-PCR:

	Relative Quantity (ΔCt)		Ct		
	Sample 1:2	Sample 1:6	Sample 1:2	Sample 1:6	Negative control
GAPDH	1	1	23.84	25.34	undetermined
PD-1	0.01	0.01	30.3	32.23	undetermined
PD-L1	0.0015	0.001	33.19	35.37	undetermined
PD-L2	0.00036	0.00029	35.25	37.11	undetermined
VISTA	0.2	0.3	26.34	26.92	undetermined

Patient samples were then studied. Significantly lower relative quantities of PD-L1 mRNA were observed in GPA patients both on conventional therapies and following B cell depletion therapy compared to healthy controls (Figure 5.2.1 B). No significant difference in mRNA encoding either PD-1 or PD-L2 was observed across four groups when whole PBMC were analysed (Figure 5.2.1 A, C). The relative quantities of mRNA encoding PD-1, PD-L1 or PD-L2 were compared to each other. Approximately 10 times more PD-1 transcripts were detected compared to PD-L1 or PD-L2 ($P<0.0001$).

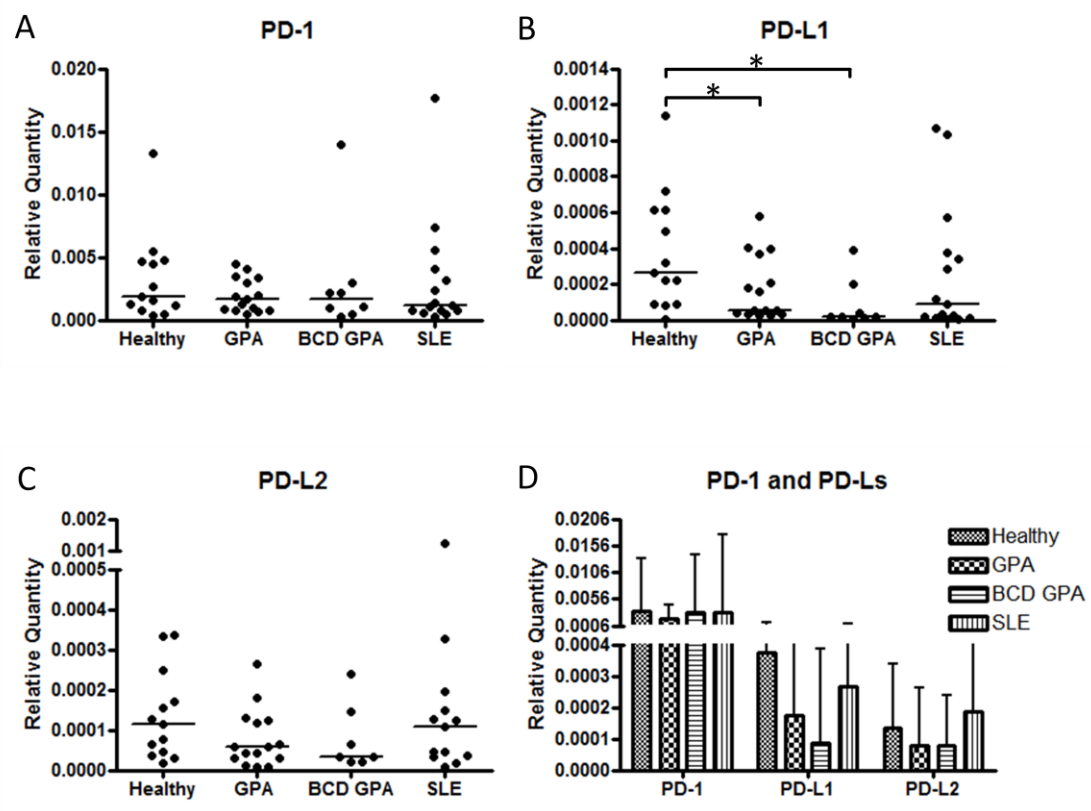

Figure 5.2.1: Relative quantities of mRNA encoding PD-1 and PD-Ls in total PBMC from healthy controls, GPA patients, B cell depleted GPA patients and SLE patients. **A.** Relative quantity of mRNA of PD-1 to GAPDH in total PBMC from healthy controls (median=0.002, range: 0.0004 to 0.013), GPA patients on conventional therapies (average=0.002, range: 0.0005 to 0.0045), GPA patients under B cell depletion therapy (median=0.0016, range: 0.0003 to 0.019) and SLE patients (median=0.0011, range: 0.0003 to 0.018). **B.** Relative quantity of mRNA encoding PD-L1 to GAPDH in total PBMC from healthy controls (average=0.00037, range: $7x10^{-6}$ to 0.0011), GPA patients on conventional therapies (average=0.00017, range: $2.5x10^{-5}$ to 0.00058) (P=0.043), GPA patients under B cell depletion therapy (median=0.00002, range: $8x10^{-6}$ to 0.00039) (P=0.015) and SLE patients (median=0.00009, range: $5.1x10^{-6}$ to 0.0011). **C.** Relative quantity of mRNA encoding PD-L2 to GAPDH in total PBMC from healthy controls (average=0.00014, range: $1.7x10^{-5}$ to 0.00034), GPA patients on conventional therapies (median=0.00006, range: $9.8x10^{-6}$ to 0.00026), GPA patients under B cell depletion therapy (median=0.00003, range: $2x10^{-5}$ to 0.00024) and SLE patients (median=0.00011, range: $9.1x10^{-6}$ to 0.0012). **D.** Comparison of relative quantities of mRNA of PD-1, PD-L1 or PD-L2 in total PBMC. Relative quantity of mRNA of PD-1 was significantly higher than that of PD-L1 in total PBMC (P<0.0001). No difference on relative quantities of mRNA of PD-L1 and PD-L2 (P=0.21).

5.2.2 Analysis of expression of PD-1, PD-L1 and PD-L2 by lymphocyte subsets and monocytes in GPA patients, healthy and disease controls

5.2.2.1 Experimental strategies for studying the expression of PD-1, PD-L1 and PD-L2 in different cell types

Since mRNA encoding PD-L1 was significantly lower in PBMC from patients with GPA, as described above, this was investigated further. PD-1 and its ligands PD-L1 and PD-L2 can be expressed on many different cell types [234] and so the expression of PD-1 and its two ligands PD-L1 and PD-L2 on lymphocyte subsets was analysed, including CD4+ T cells, CD8+ T cells and CD19+ B cells, and also CD14+ monocytes. Pilot experiments were carried out with blood samples from GPA patients on conventional therapies, healthy controls, GPA patients following B cell depletion therapy and SLE patients and were studied by flow cytometry to see if this method could be used. The gating strategies tested for flow cytometry are illustrated in figure 5.2.2 (lymphocytes) and figure 5.2.3 (monocytes). As shown in figure 5.2.2 B, PD-L1 and PD-L2 expression was very low on CD4+T cells, CD8+ T cells and B cells, and this data was therefore considered unreliable. Therefore only PD-1 expression on lymphocyte subsets and monocytes was analysed in detail by flow cytometry and will be illustrated in sections 5.2.2.2 – 5.2.2.5 below.

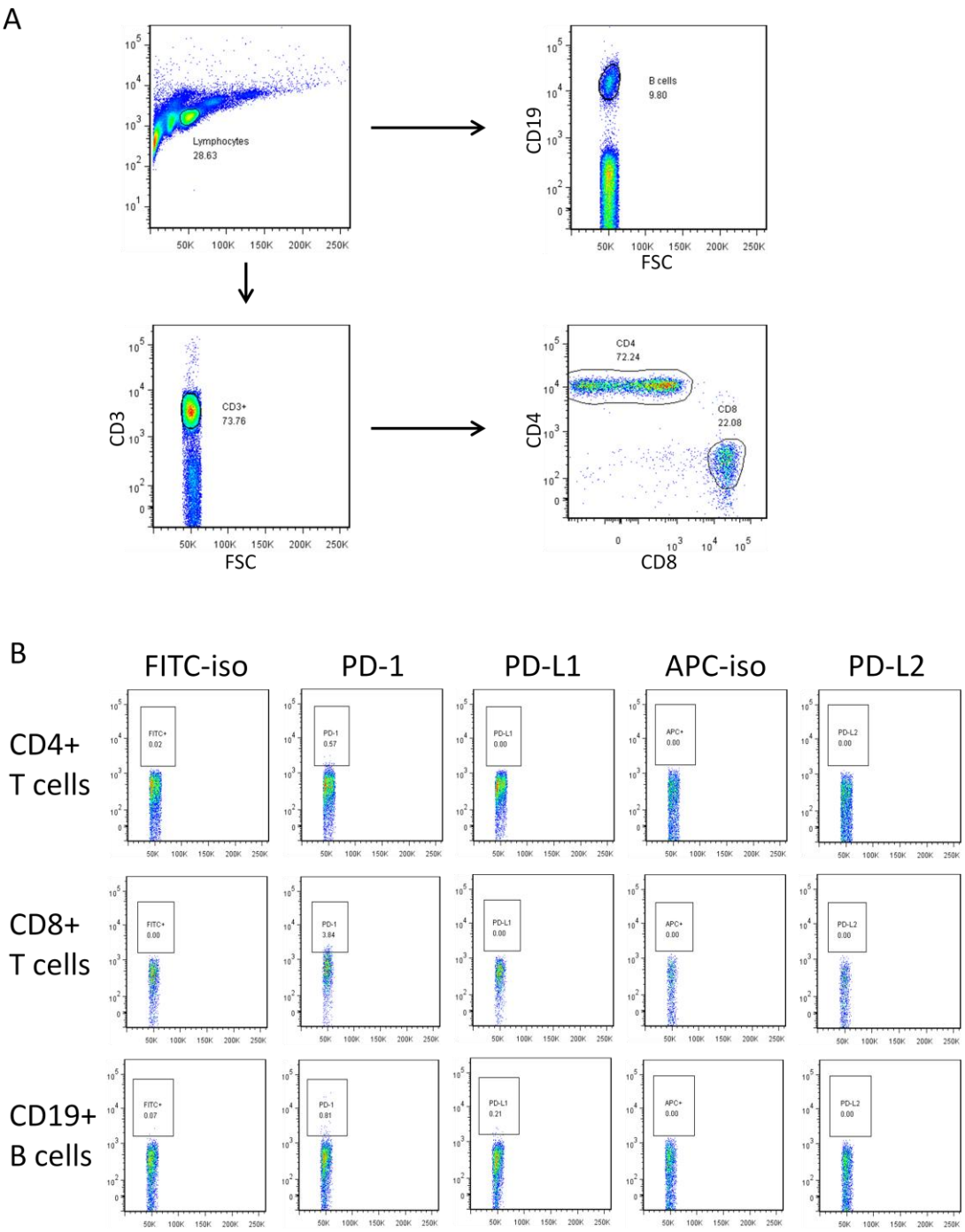

Figure 5.2.2: Expression of PD-1 and PD-Ls on different lymphocyte subsets from healthy controls, GPA patients on conventional therapies and B cell deplete GPA patients. **A and B.** Lymphocytes identified by their forward and side scatter properties were gated for CD19+ expression, CD3+CD4+ expression, or CD3+CD4- expression and then examined for the expression of PD-1, PD-L1 and PD-L2. **B.** The expression of PD-1 and PD-Ls on different lymphocyte subsets was measured according to FMO and isotype controls.

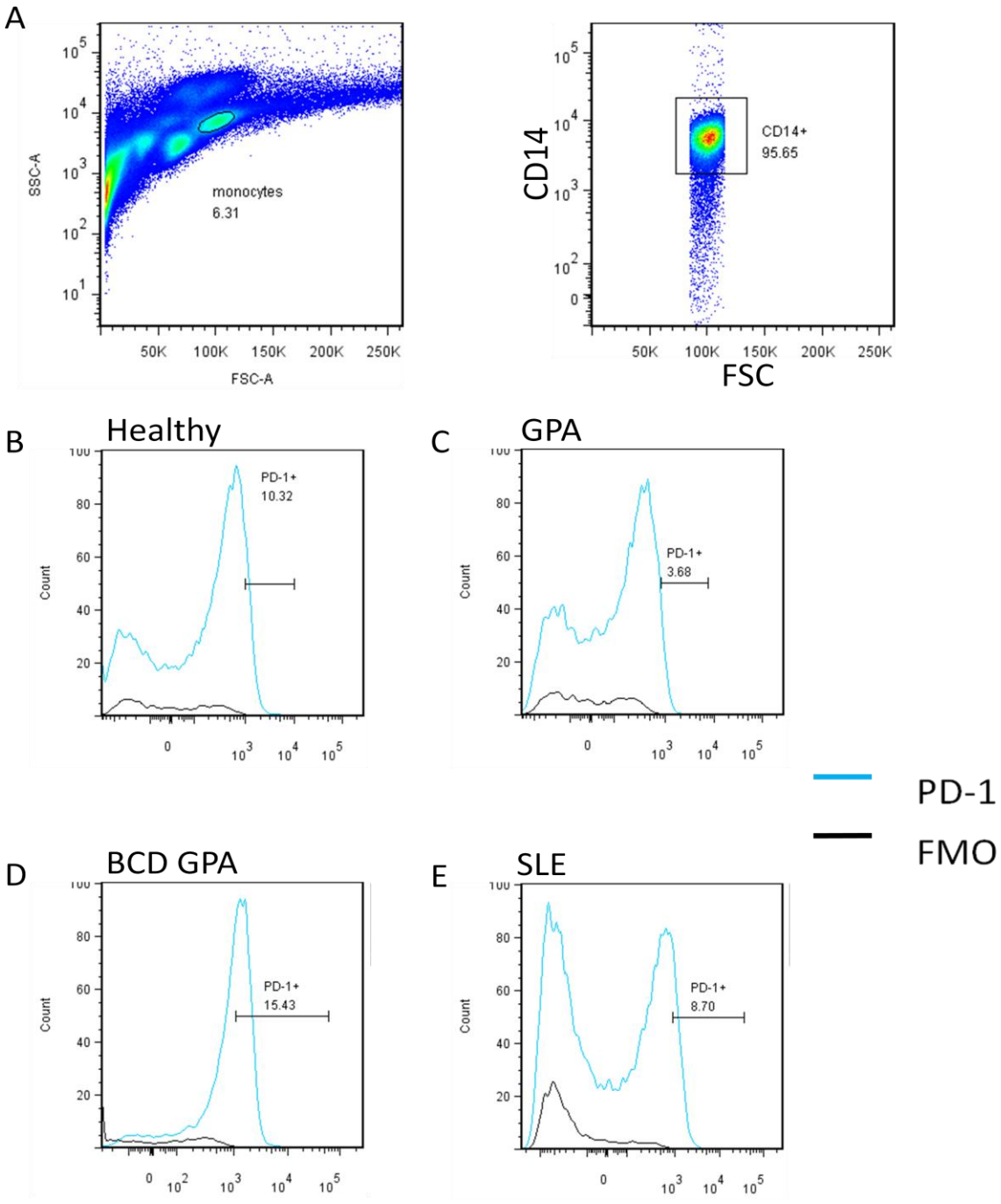

Figure 5.2.3: Expression of PD-1 on monocytes from healthy controls, GPA patients on conventional therapies, B cell depleted GPA patients and SLE patients. **A.** Monocytes identified by their forward and side scatter properties were gated for CD14+ expression to further study the expression of PD-1 on CD14+ monocytes. **B to E.** The expression of PD-1 on monocytes from healthy controls, GPA patients on conventional therapies, B cell depleted GPA patients and SLE patients was measured according to FMO controls.

RT-PCR was considered reliable for studying the expression of PD-1, PD-L1 and PD-L2 according to preliminary data (Table 5.2.4). The relative quantity of mRNA encoding PD-1 and its two ligands PD-L1 and PD-L2 in lymphocyte subsets, including CD3+CD4+ T cells, CD3+CD8+ T cells, CD19+ B cells, and CD14+ monocytes, was quantified by RT-PCR. cDNA was prepared from RNA isolated from sorted lymphocyte subsets and monocytes of GPA patients on conventional therapies, healthy controls, B cell depleted GPA patients and SLE patients. Cell isolation strategy used is shown in figure 5.2.4. Data obtained is described in the sections below. Significant findings will then be summarised in section 5.2.4.

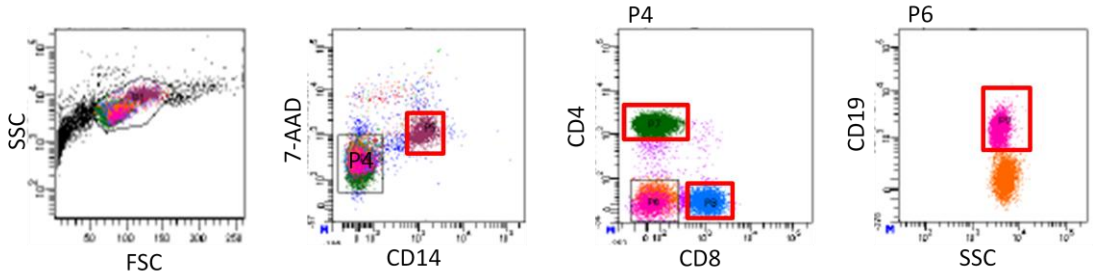

Figure 5.2.4: **Sort of different lymphocyte subsets and monocytes from healthy controls, GPA patients, B cell depleted GPA patients and SLE patients.** Monocytes identified by their forward and side scatter properties were sorted for CD14+ expression. Lymphocytes identified by their forward and side scatter properties were gated for CD14- expression and then sorted for expression of CD4+, CD8+ or CD4-CD8-CD19+ to isolate CD4+ T cells, CD8+ T cells and CD19+ B cells.

Efforts were made to ensure that the experiments in this chapter were not biased by either sample collection, or runs of the RT-PCR analysis. Blood samples were collected in mixed batches and cells were sorted over a period of three

months. RNA was isolated on freshly sorted cells and prepared into cDNA in mixed batches. Samples to be compared were then located on the same RT-PCR plate side by side where possible.

5.2.2.2 Expression of PD-1, PD-L1 and PD-L2 by CD4+ T cells in GPA patients, healthy and disease controls

PD-1 expression by CD4+ T cells from 6 patients with GPA on conventional therapies, 6 healthy controls and 4 GPA patients following B cell depletion therapy was studied by flow cytometry. The average percentage of PD-1+ cells in CD4+ T cells was 0.98 % in healthy controls. No significant difference in PD-1 expression by CD4+ T cells from healthy controls, GPA patients on conventional therapies or GPA patients following B cell depletion therapy was observed (Figure 5.2.5).

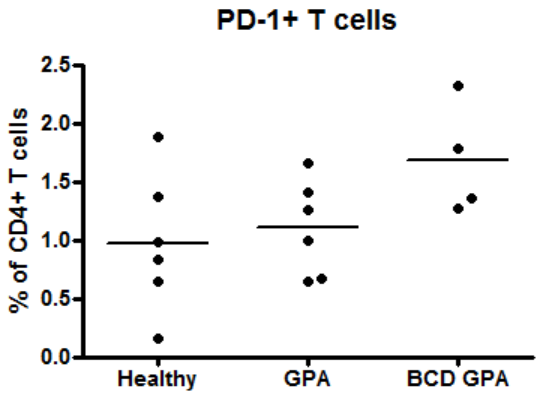

Figure 5.2.5: Expression of PD-1 on CD4+ T cells from healthy controls, GPA patients and B cell depleted GPA patients. Percentage of PD-1+ cells in CD4+ T cells from healthy controls (average=0.98, range: 0.16 to 1.88), GPA patients on conventional therapies (average=1.11, range: 0.64 to 1.66) and GPA patients following B cell depletion therapy (average=1.68, range: 1.27 to 2.32).

The relative quantity of mRNA encoding PD-1 and its ligands PD-L1 and PD-L2 in CD4+ T cells was quantified by RT-PCR. cDNA was prepared from RNA isolated from 16 GPA patients on conventional therapies, 12 healthy controls, 7 GPA patients following B cell depletion therapy and 12 SLE patients. As shown in figure 5.2.6 A, the relative quantities of PD-1 mRNA to GAPDH were widely spread in all patient groups. Although the average relative quantity of PD-1 mRNA in GPA patients following B cell depletion therapy was higher than in healthy controls and GPA patients on conventional therapies, there was no significant difference detected between these study groups.

The relative quantity of mRNA encoding PD-L1 in CD4+ T cells from GPA patients on conventional therapies was significantly lower than the relative quantity observed in cells from age and gender matched healthy controls (Figure 5.2.6 B, P=0.0024). A lower relative quantity of mRNA encoding PD-L1 in CD4+ T cells from GPA patients who had responded clinically to B cell depletion therapy compared to healthy controls was observed, but this difference was less marked than in the difference between conventionally treated GPA patients and healthy controls (Figure 5.2.6 B, P=0.046). Further comparison of the two groups of GPA patients demonstrated a significantly lower relative quantity of mRNA encoding PD-L1 in CD4+ T cells in the group on conventional therapies compared to those who were following B cell depletion therapy (Figure 5.2.6 B, P=0.016).

A significantly lower relative quantity of PD-L1 mRNA in CD4+ T cells was also detected in SLE patients when compared with healthy controls (P=0.035).

The relative quantity of mRNA encoding PD-L2 in CD4+ T cells from GPA patients on conventional therapies was significantly lower when compared to age and gender matched healthy controls (Figure 5.2.6 C, P=0.023). However, no such difference was observed in GPA patients who had responded clinically to B cell depletion therapy. Further comparison of the two groups of GPA patients demonstrated a significantly lower relative quantity of mRNA encoding PD-L2 in CD4+ T cells in the group on conventional therapies compared to those who were following B cell depletion therapy (Figure 5.2.6 C, P=0.025). No difference in the relative quantity of PD-L2 mRNA in CD4+ T cells was detected in SLE patients compared with healthy controls.

The relative quantity levels of mRNA encoding PD-1, PD-L1 and PD-L2 was further compared. As in total PBMC, the level of mRNA encoding PD-1 in CD4+ T cells was approximately 10 times more than PD-L1 (P<0.0001), which was significantly more than PD-L2 (Figure 5.2.6 D, P<0.0001).

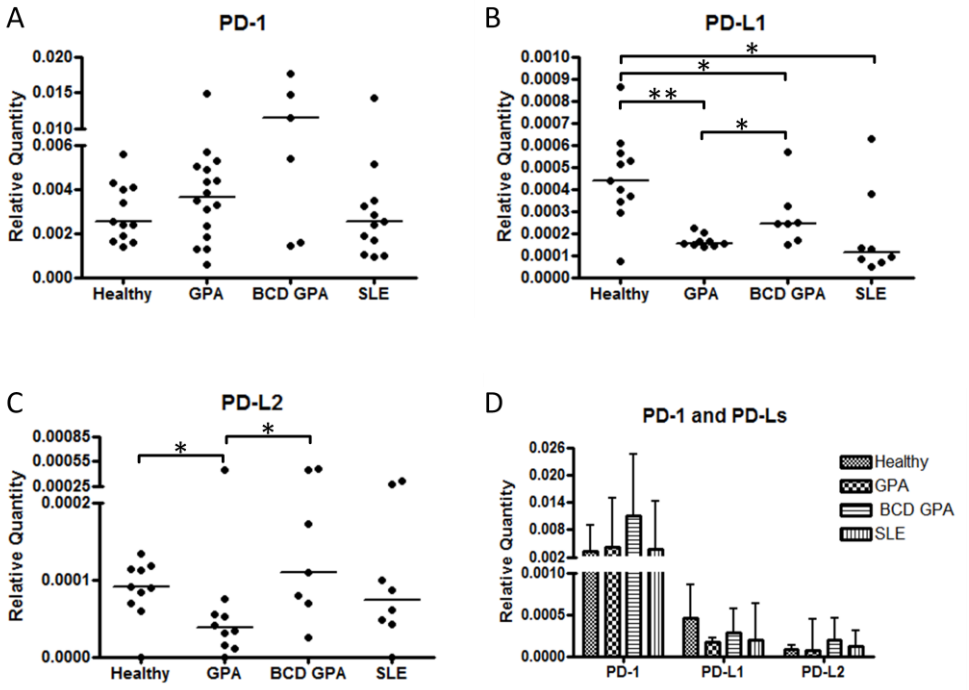

Figure 5.2.6: Relative quantities of mRNA encoding PD-1 and PD-Ls in CD4+ T cells from healthy controls, GPA patients, B cell depleted GPA patients and SLE patients. **A.** Relative quantity of mRNA of PD-1 to GAPDH in CD4+ T cells from healthy controls (median=0.0025, range: 0.0014 to 0.009), GPA patients on conventional therapies (median=0.0036, range: 0.0006 to 0.015), GPA patients under B cell depletion therapy (median=0.011, range: 0.0014 to 0.025) and SLE patients (median=0.0025, range: 0.0009 to 0.014). **B.** Relative quantity of mRNA of PD-L1 to GAPDH in CD4+ T cells from healthy controls (average=0.00045, range: $7.2x10^{-5}$ to 0.00086), GPA patients on conventional therapies (average=0.00017, range: 0.00014 to 0.00022) (P=0.0024), GPA patients under B cell depletion therapy (median=0.00025, range: 0.00015 to 0.00057) (P=0.046 and P=0.016 when compared to healthy controls and GPA patients on conventional therapies respectively) and SLE patients (median=0.00011, range: $5x10^{-5}$ to 0.00063) (P=0.035). **C.** Relative quantity of mRNA of PD-L2 to GAPDH in CD4+ T cells from healthy controls (average=0.00009, range: 0 to 0.00013), GPA patients on conventional therapies (median=0.00004, range: 0 to 0.00045) (P=0.023), GPA patients under B cell depletion therapy (median=0.00011, range: $2.5x10^{-5}$ to 0.00046) (P=0.025 when compared with GPA patients on conventional therapies) and SLE patients (average=0.00012, range: 0 to 0.00031). **D.** Comparison of relative quantities of mRNA of PD-1, PD-L1 or PD-L2 in CD4+ T cells. Relative quantity of mRNA of PD-1 was significantly higher than that of PD-L1 in CD4+ T cells (P<0.0001). Relative quantities of mRNA of PD-L1 was significantly higher than that of PD-L2 in CD4+ T cells (P<0.0001).

5.2.2.3 Expression of PD-1, PD-L1 and PD-L2 by CD8+ T cells in GPA patients, healthy and disease controls

The average percentage of cells expressing PD-1 in CD8+ T cell population was 1.52% in healthy controls. An increase in PD-1 expression on CD8+ T cells from GPA patients on conventional therapies was observed when compared to healthy controls. No such difference was observed in patients who clinically responded to B cell depletion therapy (Figure 5.2.7).

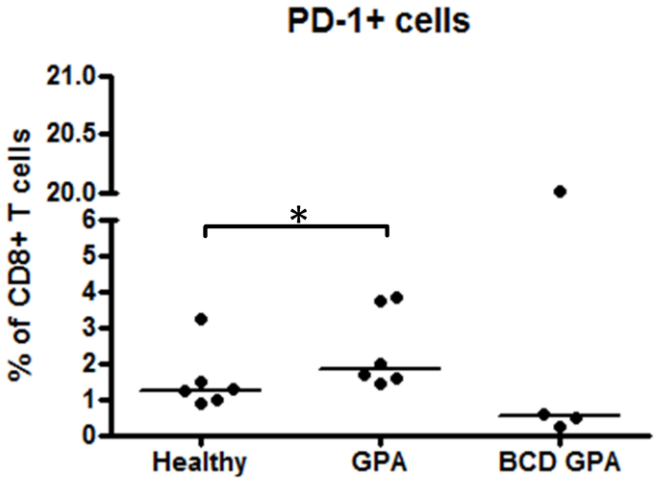

Figure 5.2.7: Expression of PD-1 on CD8+ T cells from healthy controls, GPA patients and B cell depleted GPA patients. Percentage of PD-1+ cells in CD8+ T cells from healthy controls (average=1.52, range: 0.86 to 3.23), GPA patients on conventional therapies (average=2.37, range: 1.43 to 3.81) and GPA patients under B cell depletion therapy (average=5.32, range: 0.22 to 20.01).

The relative quantity of mRNA encoding PD-1 in CD8+ T cells of 16 GPA patients on conventional therapies, 13 healthy controls, 7 GPA patients following B cell depletion therapy and 11 SLE patients was quantified by RT-PCR. No

significant difference was detected in either group of GPA patients or SLE patients compared with healthy controls (Figure 5.2.8 A).

The relative quantity of mRNA encoding PD-L1 in CD8+ T cells was found to be lower in GPA patients and SLE patients compared to healthy controls. A tendency for the relative quantity of PD-L1 mRNA in GPA patients on conventional therapies to be lower than those who were following B cell depletion therapy was observed. However, the differences here were not significant (Figure 5.2.8 B).

The relative quantity of mRNA encoding PD-L2 in CD8+ T cells from GPA patients on conventional therapies was significantly lower compared to age and gender matched healthy controls (Figure 5.2.8 C, P=0.029). However, there was no difference in the relative quantity of mRNA encoding PD-L2 in CD8+ T cells from GPA patients following B cell depletion therapy compared to age and gender matched healthy controls. The relative quantity of mRNA encoding PD-L2 on CD8+ T cells was the same in the two groups of GPA patients. No difference in the relative quantity of PD-L2 mRNA in CD8+ T cells was detected in SLE patients compared with healthy controls.

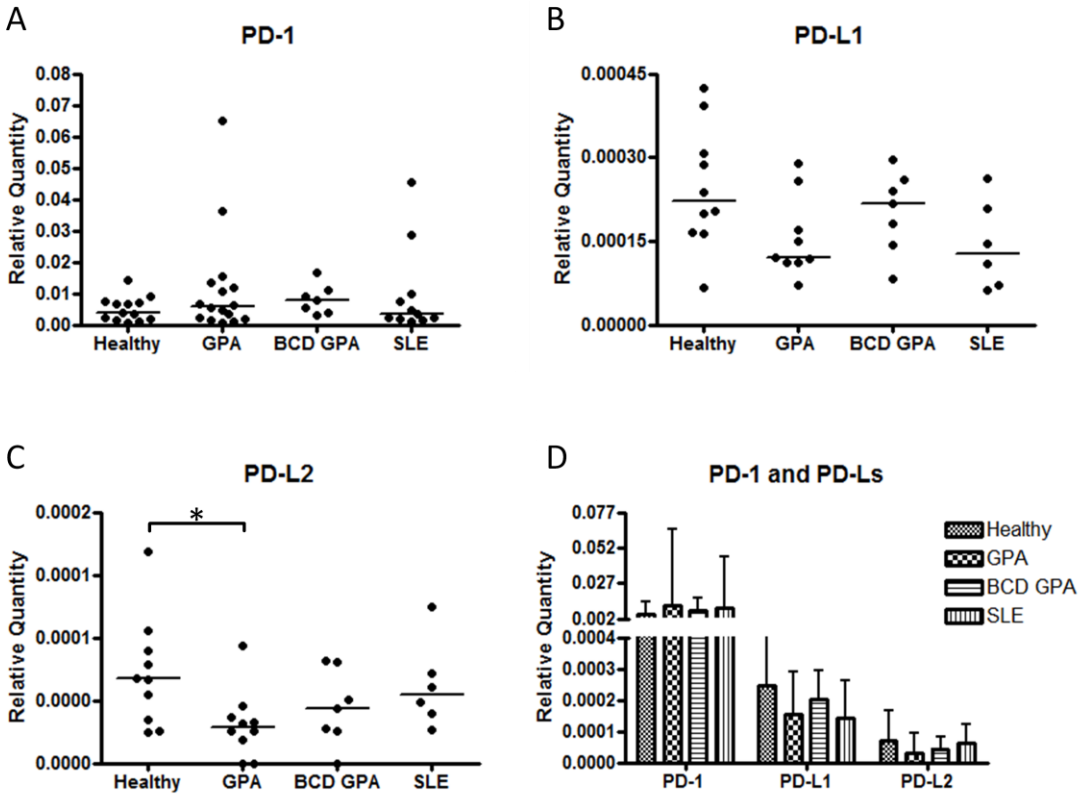

Figure 5.2.8: Relative quantities of mRNA encoding PD-1 and PD-Ls in CD8+ T cells from healthy controls, GPA patients, B cell depleted GPA patients and SLE patients. **A.** Relative quantity of mRNA of PD-1 to GAPDH in CD8+ T cells from healthy controls (average=0.0051, range: 0.0007 to 0.014), GPA patients on conventional therapies (median=0.0058, range: 0.0007 to 0.065), GPA patients under B cell depletion therapy (median=0.0077, range: 0.0029 to 0.017) and SLE patients (median=0.0036, range: 0.001 to 0.046). **B.** Relative quantity of mRNA of PD-L1 to GAPDH in CD8+ T cells from healthy controls (average=0.00024, range: 0.00006 to 0.00042), GPA patients on conventional therapies (average=0.00016, range: 0.00007 to 0.00029), GPA patients under B cell depletion therapy (average=0.00020, range: 0.00008 to 0.0003) and SLE patients (average=0.00014, range: 0.00006 to 0.00026). **C.** Relative quantity of mRNA of PD-L2 to GAPDH in CD8+ T cells from healthy controls (average=0.00007, range: 0.00002 to 0.00017), GPA patients on conventional therapies (median=0.00003, range: 0 to 0.00009) (P=0.0288), GPA patients under B cell depletion therapy (median=0.00004, range: 0 to 0.00008) and SLE patients (median=0.00005, range: 0.00003 to 0.00012). **D.** Comparison of relative quantities of mRNA of PD-1, PD-L1 or PD-L2 in CD8+ T cells. Relative quantity of mRNA of PD-1 was significantly higher than that of PD-L1 in CD8+ T cells (P<0.0001). Relative quantities of mRNA of PD-L1 was significantly higher than that of PD-L2 in CD8+ T cells (P<0.0001).

The level of mRNA encoding PD-1 in CD8+ T cells was significantly higher than that encoding PD-L1 ($P<0.0001$), which was significantly higher than that encoding PD-L2 (Figure 5.2.8 D, $P<0.0001$).

5.2.2.4 Expression of PD-1, PD-L1 and PD-L2 by CD19+ B cells in GPA patients, healthy and disease controls

The percentage of PD-1+ cells in CD19+ B cells was similar to that in CD4+ T cells and CD8+ T cells. The average percentage was 1.19% in healthy controls (Figure 5.2.9 A). No difference in the proportion of CD19+ B cells expressing PD1 was observed in GPA patients on conventional therapies compared to healthy controls. There were no PD-1+CD19+ B cells detected in GPA patients who had clinically responded to B cell depletion therapy, but the B cell numbers in this group were very low (Figure 5.2.9 B).

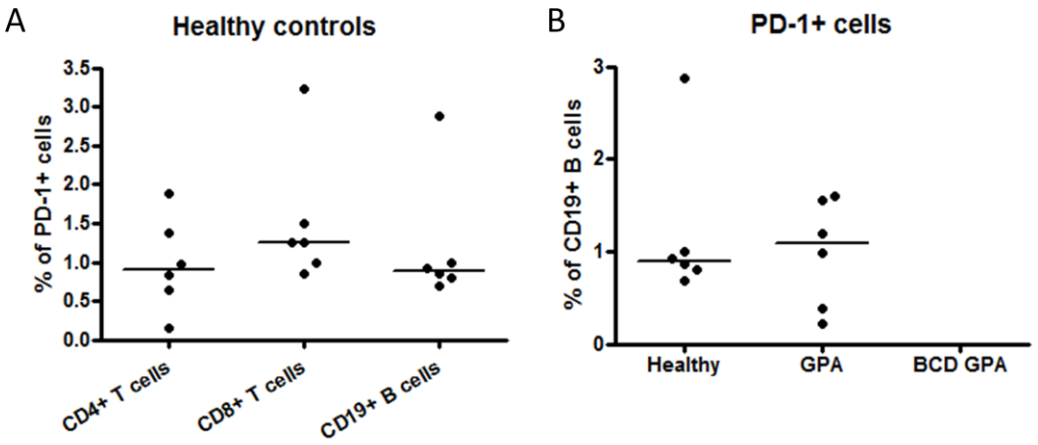

Figure 5.2.9: Expression of PD-1 on CD19+ B cells from healthy controls, GPA patients and B cell depleted GPA patients. **A.** Comparison of PD-1 expression in CD4+ T cells (average=0.98, range: 0.16 to 1.88), CD8+ T cells (average=1.52, range: 0.86 to 3.23), and CD19+ B cells (average=1.19, range=0.69 to 2.88) from healthy controls.

B. Percentage of PD-1+ cells in CD19+ B cells from healthy controls (average=1.19, range=0.69 to 2.88), GPA patients on conventional therapies (average=0.99, range: 0.22 to 1.6). No PD-1+ CD19+ B cell was detected in GPA patients under B cell depletion therapy.

As there were very few CD19+ B cell in GPA patients following B cell depletion therapy, only mRNA from GPA patients on conventional therapies, healthy controls and SLE patients was quantified by RT-PCR. The relative quantity of mRNA encoding PD-1 in CD19+ B cells of GPA patients was quantified, and no significant difference was detected compared with healthy controls (Figure 5.2.10 A). The range of values for relative quantity of PD-1 mRNA in CD19+ B cells from SLE patients was wider than from healthy controls and GPA patients, but the difference was not significant.

No significant difference was observed in the relative quantity of mRNA encoding PD-L1 in CD19+ B cells from GPA patients on conventional therapies compared to age and gender matched healthy controls (Figure 5.2.10 B). No difference in the relative quantity of PD-L1 mRNA in CD19+ B cells was detected in SLE patients compared with healthy controls. Similarly, no significant difference in the relative quantity of mRNA encoding PD-L2 in CD19+ B cells was detected between any groups analysed (Figure 5.2.10 C).

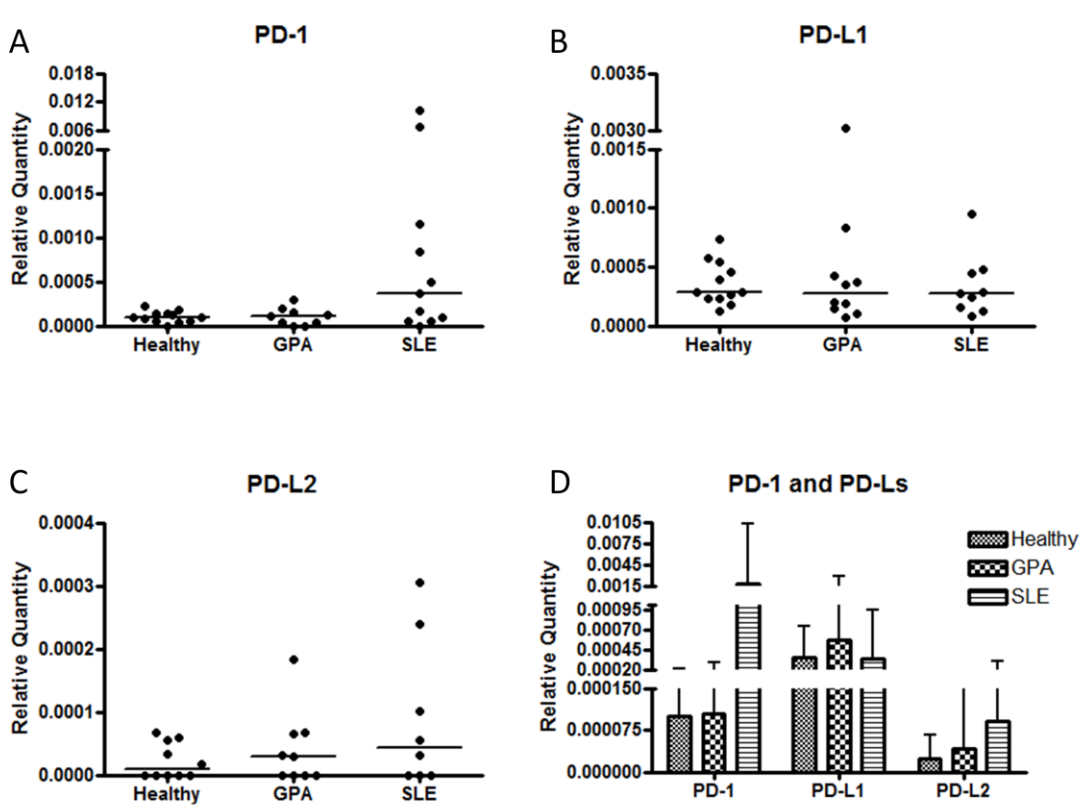

Figure 5.2.10: Relative quantities of mRNA encoding PD-1 and PD-Ls in CD19+ B cells from healthy controls, GPA patients and SLE patients. **A.** Relative quantity of mRNA of PD-1 to GAPDH in CD19+ B cells from healthy controls (average=0.0001, range: 0.00005 to 0.00022), GPA patients on conventional therapies (average=0.00011, range: 0.00002 to 0.0003) and SLE patients (average=0.0018, range: 0.00006 to 0.01). **B.** Relative quantity of mRNA of PD-L1 to GAPDH in CD19+ B cells from healthy controls (average=0.00036, range: 0.00013 to 0.00074), GPA patients on conventional therapies (average=0.00057, range: 0.00007 to 0.003) and SLE patients (average=0.00034, range: 0.00008 to 0.00095). **C.** Relative quantity of mRNA of PD-L2 to GAPDH in CD19+ B cells from healthy controls (average=0.00002, range: 0 to 0.00007), GPA patients on conventional therapies (average=0.00004, range: 0 to 0.00018) and SLE patients (average=0.00009, range: 0 to 0.00031). **D.** Comparison of relative quantities of mRNA of PD-1, PD-L1 or PD-L2 in CD19+ B cells. Relative quantity of mRNA of PD-L1 was significantly higher than that of PD-1 in CD19+ B cells from healthy controls and GPA patients ($P<0.0001$). Relative quantities of mRNA of PD-1 was significantly higher than that of PD-L2 in CD19+ B cells from all groups studied ($P=0.006$).

The relative quantity of mRNA encoding PD-1, PD-L1 and PD-L2 in CD19+ B cells from all studied groups was further compared. Different from that in total PBMC, CD4+ T cells and CD8+ T cells, the level of mRNA encoding PD-L1 was highest in CD19+ B cells from GPA patients and healthy controls ($P<0.0001$ when compared to either PD-1 or PD-L2), while the relative quantity of mRNA encoding PD-1 was higher than that encoding PD-L2 in all groups (Figure 5.2.10 D, $P=0.0003$). The range of data was very wide, especially for the SLE group.

5.2.2.5 Expression of PD-1, PD-L1 and PD-L2 by CD14+ monocytes in GPA patients, healthy and disease controls

The average of CD14+ monocytes expressing PD-1 was 10.92% (healthy controls), 10.98% (GPA patients under B cell depletion therapy) and 10.14% (SLE patients). These percentages were significantly higher than the 3.76% observed in GPA patients on conventional therapies (Figure 5.2.11).

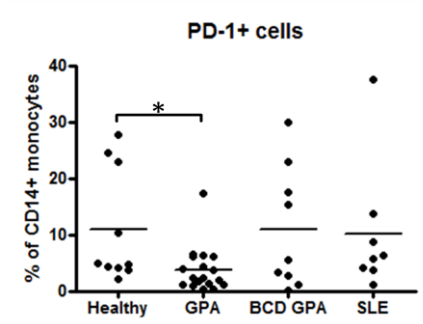

Figure 5.2.11: Expression of PD-1 on CD14+ monocytes from healthy controls, GPA patients, B cell depleted GPA patients and SLE patients. Percentage of PD-1+ cells in CD14+ monocytes from healthy controls (average=10.92, range: 2.09 to 27.77), GPA patients on conventional therapies (average=3.76, range: 0.36 to 17.27) ($P=0.019$), GPA patients under B cell depletion therapy (average=10.98, range: 0.17 to 29.99) and SLE patients (average=10.14, range: 1.11 to 37.54).

The relative quantity of mRNA encoding PD-1 in CD14+ monocytes was quantified by RT-PCR. No significant difference was detected in either of the two groups of GPA patients or SLE patients compared with healthy controls (Figure 5.2.12 A).

The relative quantity of mRNA encoding PD-L1 in CD14+ monocytes was quantified by RT-PCR. A lower relative quantity of mRNA encoding PD-L1 was observed in GPA patients on conventional therapies compared to healthy controls (Figure 5.2.12 B, P=0.052). A significantly lower relative quantity of mRNA encoding PD-L1 in CD14+ monocytes was observed in GPA patients who had clinically responded to B cell depletion therapy, compared to healthy controls (P=0.023). Further comparison of the two groups of GPA patients did not illustrate difference in the relative quantity of mRNA encoding PD-L1 in CD14+ monocytes (Figure 5.2.12 B, P=0.49). No difference of relative quantity of PD-L1 mRNA in CD14+ monocytes from SLE patients was detected compared to healthy controls (P=0.5).

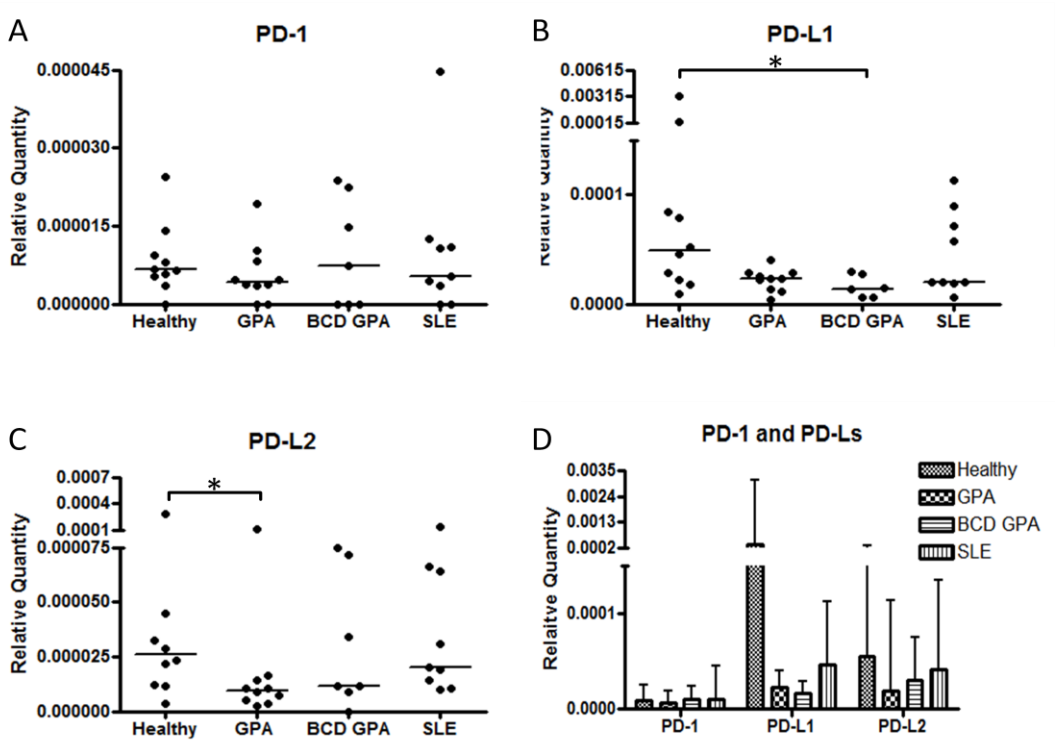

Figure 5.2.12: Relative quantities of mRNA encoding PD-1 and PD-Ls in CD14+ monocytes from healthy controls, GPA patients, B cell depleted GPA patients and SLE patients. **A.** Relative quantity of mRNA of PD-1 to GAPDH in CD14+ monocytes from healthy controls (median=$7x10^{-6}$, range: 0 to 0.000025), GPA patients on conventional therapies (median=$4x10^{-6}$, range: 0 to 0.000019), GPA patients under B cell depletion therapy (median=$7x10^{-6}$, range: 0 to 0.000024) and SLE patients (median=$5x10^{-6}$, range: 0 to 0.000045). **B.** Relative quantity of mRNA of PD-L1 to GAPDH in CD14+ monocytes from healthy controls (median=0.00049, range: $9.6x10^{-6}$ to 0.0031), GPA patients on conventional therapies (average=0.00002, range: $3.7x10^{-6}$ to 0.00004) (P=0.052), GPA patients under B cell depletion therapy (median=0.00001, range: $5.8x10^{-6}$ to 0.00003) (P=0.023 when compared to healthy controls) and SLE patients (average=0.00005, range: $5.6x10^{-6}$ to 0.00011). **C.** Relative quantity of mRNA of PD-L2 to GAPDH in CD14+ monocytes from healthy controls (median=0.00003, range: $3.4x10^{-6}$ to 0.00028), GPA patients on conventional therapies (median=0.00001, range: $2.7x10^{-6}$ to 0.00011) (P=0.029), GPA patients under B cell depletion therapy (median=0.00001, range: 0 to 0.00007) and SLE patients (median=0.00002, range: 0.00001 to 0.00013). **D.** Comparison of relative quantities of mRNA of PD-1, PD-L1 or PD-L2 in CD14+ monocytes. Relative quantity of mRNA of PD-L1 was higher than that of PD-L2 in CD14+ monocytes (P=0.14). Relative quantity of mRNA of PD-L2 was significantly higher than that of PD-1 in CD14+ monocytes (P<0.0001).

A significantly lower relative quantity of mRNA encoding PD-L2 in CD14+ monocytes was observed in GPA patients on conventional therapies, compared to age and gender matched healthy controls (Figure 5.2.12 C, P=0.029). However, no significant difference in the relative quantity of mRNA encoding PD-L2 in CD14+ monocytes was observed in GPA patients following B cell depletion therapy, compared to age and gender matched healthy controls (P=0.54). Further comparison of the two groups of GPA patients did not illustrate a significant difference in the relative quantity of mRNA encoding PD-L2 in CD14+ monocytes (P=0.31). No difference in the relative quantity of PD-L2 mRNA in CD14+ monocytes was detected in SLE patients compared with healthy controls (Figure 5.2.12 C, P=0.84).

The relative quantity levels of mRNA encoding PD-1, PD-L1 and PD-L2 in CD14+ monocytes from all studied groups were further compared. Different from that in total PBMC and different lymphocyte subsets, the levels of mRNA encoding PD-L1 was higher than PD-L2 mRNA (P=0.14), and both mRNA encoding PD-L1 and PD-L2 was significantly higher than that encoding PD-1 in all groups (Figure 5.2.12 D, P<0.0001 respectively).

5.2.2.6 Comparison of mRNA relative quantities of PD-1, PD-L1 and PD-L2 by different cell types in GPA patients, healthy and disease controls

The relative quantity of mRNA encoding PD-1, PD-L1 and PD-L2 was quantified in CD4+ T cells, CD8+ T cells, CD19+ B cells and CD14+ monocytes. In the sections above differences within a cell type were analysed. In this section, relative mRNA levels between cell types were compared. The relative quantity of PD-1 mRNA was highest in CD8+ T cells, followed by CD4+ T cells then CD19+ B cells in all patient and healthy groups. The relative quantity of PD-1 mRNA in CD14+ monocytes was more than 10 times lower than in other cell types ($P<0.0001$) in all groups studied (Figure 5.2.13 A).

The relative quantity of PD-L1 mRNA in different cell types in healthy controls was compared. No significant difference was observed in CD4+ T cells and CD19+ B cells from healthy control group (Figure 5.2.13 B). Compared to CD4+ T cells, a significantly lower relative quantity of PD-L1 mRNA in CD8+ T cells was observed in healthy controls ($P=0.0083$), while the average relative quantity of PD-L1 mRNA in monocytes was significantly higher than in CD8+ T cells from healthy controls ($P=0.012$).

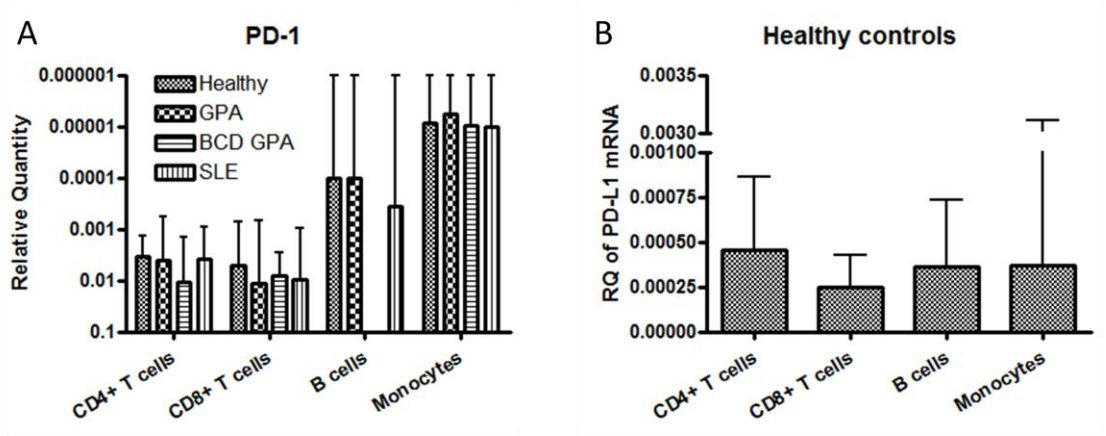

Figure 5.2.13: Comparison of relative quantities of mRNA encoding PD-1 and PD-L1 in different immune cell types. **A.** Relative quantity of mRNA of PD-1 in CD4+ T cells (average=0.0048, range: 0.00058 to 0.025), CD8+ T cells (average=0.0089, range: 0.00069 to 0.065), CD19+ B cells (average=0.0007, range: 0 to 0.01) ($P<0.0001$ when compared with that in CD8+ T cells) and monocytes (average=$8x10^{-6}$, range: 0 to 0.00004) ($P<0.0001$ when compared with that in B cells) from all groups. **B.** Relative quantity of mRNA of PD-L1 in total PBMC (average=0.00037, range: $7x10^{-6}$ to 0.0011), CD4+ T cells (average=0.00045, range: $7.2x10^{-5}$ to 0.00086), CD8+ T cells (average=0.00024, range: 0.00006 to 0.00042), CD19+ B cells (average=0.00036, range: 0.00013 to 0.00074) and monocytes (average=0.00037, range: $9.6x10^{-6}$ to 0.0031) from healthy controls.

The relative quantity of PD-L2 mRNA in different cell types was compared according to different patient groups. A decreased tendency of relative quantity of mRNA encoding PD-L2 was observed in the subsequence of CD4+ T cells, CD8+ T cells, CD14+ monocytes and then CD19+ B cells from healthy controls (Figure 5.2.14 A). However, in GPA patients on conventional therapies and SLE patients, this tendency was CD4+ T cells, followed by CD19+ B cells then CD8+ T cells and then monocytes (Figure 5.2.14 B and D). In GPA patients following B cell

depletion therapy, the relative quantity of PD-L2 mRNA was highest in CD4+ T cells, followed by CD8+ T cells and then CD14+ monocytes (Figure 5.2.14 C).

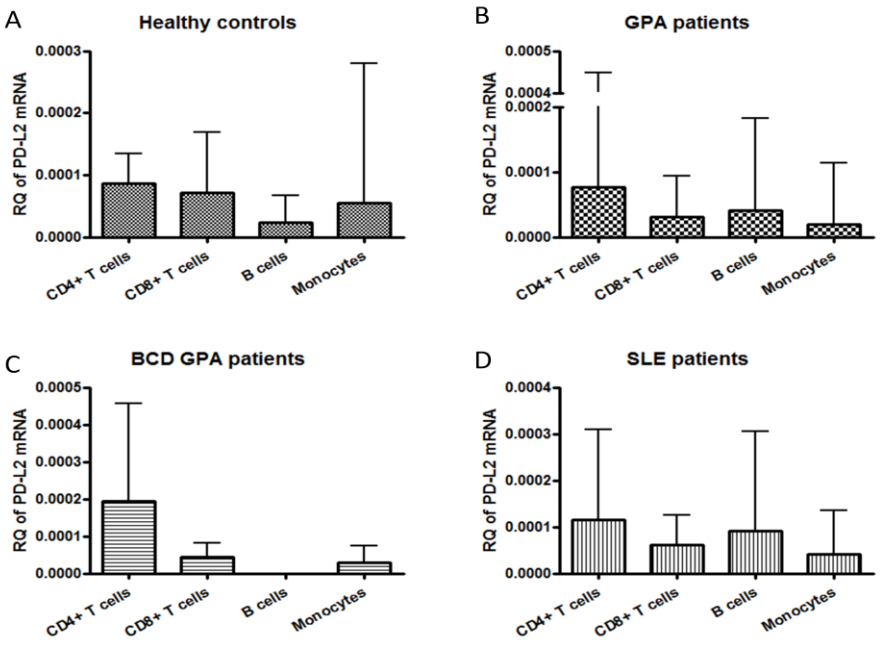

Figure 5.2.14: Comparison of relative quantities of mRNA encoding PD-L2 in different immune cell types. **A.** In healthy controls, the relative quantity of mRNA of PD-L2 in total PBMC (average=0.00014, range: $1.7x10^{-5}$ to 0.00034), CD4+ T cells (average=0.00009, range: 0 to 0.00013), CD8+ T cells (average=0.00007, range: 0.00002 to 0.00017), CD19+ B cells (average=0.00002, range: 0 to 0.00007) and monocytes (average=0.00005, range: $3.4x10^{-6}$ to 0.00028). **B.** In GPA patients on conventional therapies, the relative quantity of mRNA of PD-L2 in total PBMC (average=0.00008, range: $9.8x10^{-6}$ to 0.00026), CD4+ T cells (average=0.00008, range: 0 to 0.00045), CD8+ T cells (average=0.00003, range: 0 to 0.00009), CD19+ B cells (average=0.00004, range: 0 to 0.00018) and monocytes (average=0.00002, range: $2.7x10^{-6}$ to 0.00011). **C.** In GPA patients under B cell depletion therapy, the relative quantity of mRNA of PD-L2 in total PBMC (average=0.00008, range: $2x10^{-5}$ to 0.00024), CD4+ T cells (average=0.00019, range: $2.5x10^{-5}$ to 0.00046), CD8+ T cells (average=0.00004, range: 0 to 0.00008) and monocytes (average=0.00005, range: 0 to 0.00007). **D.** In SLE patients, the relative quantity of mRNA of PD-L2 in total PBMC (average=0.00019, range: $9.1x10^{-6}$ to 0.0012), CD4+ T cells (average=0.00012, range: 0 to 0.00031), CD8+ T cells (average=0.00006, range: 0.00003 to 0.00012), CD19+ B cells (average=0.00009, range: 0 to 0.00031) and monocytes (average=0.00004, range: 0.00001 to 0.00013).

5.2.3 Expression of VISTA by PBMC, different lymphocyte subsets and monocytes in GPA patients, healthy and disease controls

The relative quantity of mRNA encoding VISTA in PBMC, CD4+ T cells, CD8+ T cells, CD19+ B cells and CD14+ monocytes was quantified by RT-PCR. cDNA was prepared from RNA isolated from 10 GPA patients on conventional therapies, 10 healthy controls, 7 GPA patients following B cell depletion therapy and 9 SLE patients. Consistent with results from the test experiment shown in table 5.2.1, the relative quantity of mRNA encoding VISTA was much higher than that encoding PD-1, PD-L1 and PD-L2 in all cell types studied (Figure5.2.15).

There was no significant difference in the relative quantity of VISTA mRNA in whole PBMC, CD4+ T cells, CD8+ T cells, CD19+ B cells and monocytes from GPA patients on conventional therapies, GPA patients following B cell depletion therapy and SLE patients when compared with healthy controls.

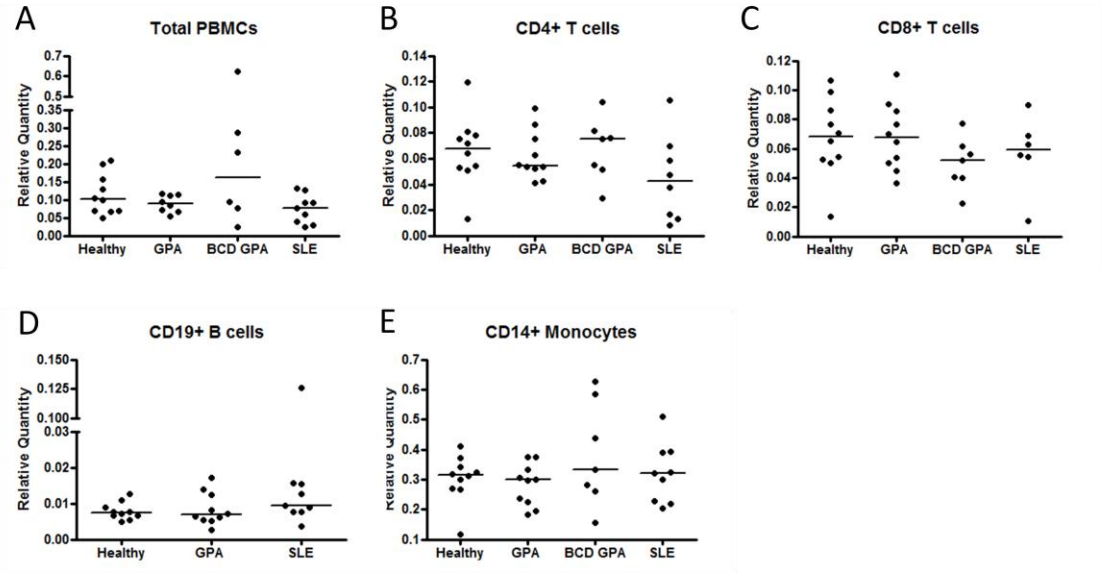

Figure 5.2.15: Relative quantities of mRNA encoding VISTA in different lymphocyte subsets and monocytes from healthy controls, GPA patients, B cell depleted GPA patients and SLE patients. **A.** Relative quantity of mRNA of VISTA in total PBMC from healthy controls (average=0.12, range: 0.049 to 0.21), GPA patients on conventional therapies (average=0.09, range: 0.054 to 0.12), GPA patients under B cell depletion therapy (average=0.22, range: 0.025 to 0.62) and SLE patients (average=0.075, range: 0.024 to 0.13). **B.** Relative quantity of mRNA of VISTA in CD4+ T cells from healthy controls (average=0.066, range: 0.013 to 0.12), GPA patients on conventional therapies (average=0.062, range: 0.041 to 0.099), GPA patients under B cell depletion therapy (average=0.068, range: 0.029 to 0.1) and SLE patients (average=0.045, range: 0.0084 to 0.11). **C.** Relative quantity of mRNA of VISTA in CD8+ T cells from healthy controls (average=0.068, range: 0.013 to 0.11), GPA patients on conventional therapies (average=0.068, range: 0.037 to 0.11), GPA patients under B cell depletion therapy (average=0.05, range: 0.023 to 0.077) and SLE patients (average=0.057, range: 0.011 to 0.09). **D.** Relative quantity of mRNA of VISTA in CD19+ B cells from healthy controls (average=0.0079, range: 0.0049 to 0.013), GPA patients on conventional therapies (average=0.0085, range: 0.0028 to 0.017), and SLE patients (median=0.0094, range: 0.0036 to 0.13). **E.** Relative quantity of mRNA of VISTA in CD14+ monocytes from healthy controls (median=0.31, range: 0.12 to 0.41), GPA patients on conventional therapies (average=0.28, range: 0.18 to 0.38), GPA patients under B cell depletion therapy (median=0.33, range: 0.16 to 0.63) and SLE patients (average=0.32, range: 0.2 to 0.51).

The relative quantity of mRNA encoding VISTA in different cell types was further compared. The relative amount of mRNA transcripts encoding VISTA was highest in monocytes, which was approximately 5 times more than in CD4+ T cells and CD8+ T cells (Figure 5.2.16, P<0.0001 respectively), while the relative quantity of VISTA mRNA was lowest in B cells which was 1/10 of that in CD4+ T cells (Figure 5.2.16, P<0.0001).

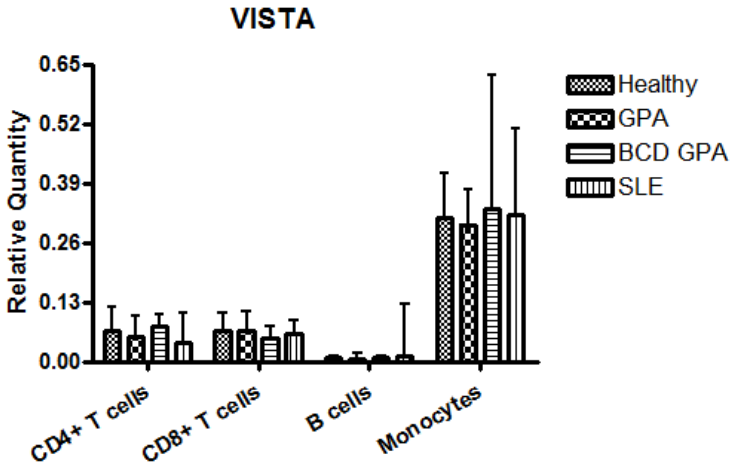

Figure 5.2.16: Comparison of relative quantities of mRNA encoding VISTA in different lymphocyte subsets and monocytes. Relative quantity of mRNA of VISTA in CD4+ T cells (average=0.06, range: 0.0084 to 0.12), CD8+ T cells (average=0.062, range: 0.011 to 0.11), CD19+ B cells (average=0.013, range: 0.0028 to 0.13) (P<0.0001 when compared with that in CD4+ T cells) and monocytes (average=0.32, range: 0.12 to 0.63) (P<0.0001 when compared with any of that in CD4+ T cells, CD8+ T cells and CD19+ B cells) from all groups.

5.2.4 Main findings in this chapter:

- Higher proportion of CD8+ T cells expressing PD-1 and lower percentage of CD14+ monocytes expressing PD-1 was observed in GPA patients on conventional therapies compared to healthy controls.
- Lower relative quantity of mRNA encoding PD-L1 in whole PBMC, CD4+ T cells and CD14+ monocytes was observed in GPA patients on conventional therapies and GPA patients following B cell depletion therapy compared to healthy controls.
- Lower relative quantity of mRNA encoding PD-L2 in CD4+ T cells, CD8+ T cells and CD14+ monocytes was observed in GPA patients on conventional therapies compared to healthy controls.
- No significant difference in and relative quantity of mRNA encoding PD-1 or VISTA was found in all cell types studied.

5.3 Discussion

5.3.1 PD-1 in GPA

The relative quantities of PD-1 mRNA in circulating cells from GPA patients has been described for the first time in this thesis. A higher percentage of CD8+ T cells expressing PD-1 and a lower percentage of CD14+ monocytes expressing PD-1 were observed in GPA patients on conventional therapies compared to healthy controls. No difference was observed in proportion of PD-1+cells in CD4+ T cells and CD19+ B cells between GPA patients and healthy controls. No significant difference in the relative quantity of mRNA encoding PD-1 was found in all cell types studied between GPA patients and healthy controls.

A significantly increased proportion of PD-1+ CD4+ T cells was observed in peripheral blood from GPA patients (average: 1.23, range: 0.7 to 2.85) compared to healthy controls (average: 0.69, range: 0.47 to 0.99) in chapter 4. However, this difference was not seen the experiments carried out for the chapter, although the percentage of PD-1+CD4+ T cells (GPA: average: 1.11, range: 0.64 to 1.66; healthy: average: 0.98, range: 0.16 to 1.88) was similar to that in chapter 4. Differences between the data might due to different staining Abs used or different patient cohorts. PD-1 expressing CD4+ T cells in GPA patients have been discussed in chapter 4.

Changes in PD-1 expression by different subsets of lymphocytes in human diseases have been observed before. Although low PD-1 expression was observed on un-stimulated CD8+ T cells in this study, PD-1 expression was reported to be up-regulated on CD8+ T cells from human blood after activation in vitro [417]. Other studies have reported PD-1 expression on peripheral lymphocytes from patients with autoimmune diseases. Fewer PD-1 expressing CD4+ T cells and CD8+ T cells in peripheral blood, and lower expression level of PD-1 on antigen experienced T cells was observed in patients with SLE [418]. Higher percentage of PD-1+ CD4+ T cells was observed in blood from patients with ankylosing spondylitis, and the proportion of PD-1 expressing T cells was inversely correlated with severity of disease outcomes [419]. One study has reported that PD-1 expression by peripheral blood T cells was undetectable, while PD-1+CD4+ T cells were enriched in synovial fluid from patients with rheumatoid arthritis [420]. PD-1 expression on CD4+ and CD8+ T cells was observed to be up-regulated in patients with stable but not active multiple sclerosis after stimulation [421]. Therefore the increased proportion of PD-1 expressing CD8+ T cells and decreased proportion of PD-1 expressing monocytes in peripheral blood from GPA patients may contribute to failure of immune tolerance.

Animal models have identified an association between PD-1 expression and autoimmune diseases. Studies have shown that mice with PD-1 deficiency can

develop various autoimmune diseases. PD-1 deficiency in different mouse models was reported to develop lupus-like autoimmune diseases [413], and more severe autoimmune disease [306, 307], diabetes [414, 422] and Ag-induced arthritis [423]. A previous study indicated that the level of PD-1 transcripts is not consistent with the level of PD-1 protein expression in mice [239]. Although no difference in PD-1 mRNA in monocytes was observed in GPA patients, the deficiency of PD-1 protein expressed on monocytes in GPA patients may contribute to the autoimmune responses through providing less suppression.

A previous study in mice has indicated that expression of PD-1 alone does not trigger apoptosis [239]. Interaction between PD-1 and its ligands has been shown to be related to autoimmune diseases in different animal models. Ligation of PD-1 expressed on activated lymphocytes with PD-L1 in addition to blocking ICOS-mediated co-stimulation, was shown to suppress the development of a lupus-like syndrome and nephritis in an autoimmune mouse model [424]. Blockade of the PD-1/PD-L1 pathway was demonstrated to accelerate type 1 diabetes in NOD mice [415], as well as break CD4+ T cell tolerance in NOD mice achieved by treatment with insulin [416]. Ligation of PD-1 with PD-L1/Fc fusion protein was reported to result in fewer infiltrated inflammatory cells and effectively improve disease in rat models for experimental autoimmune glomerulonephritis [425]. Autoreactive B cells in mice were demonstrated to

be suppressed by PD-1 ligation through PD-L1 and PD-L2 [284]. Therefore, although no difference of mRNA encoding PD-1 in lymphocytes and monocytes was observed from GPA patients in this thesis, PD-1/PD-L pathways might still contribute to the pathogenesis of GPA due to the deficiency of PD-1 ligands in different cell subsets.

5.3.2 PD-L1 in GPA

The relative quantity of PD-L1 in total PBMC, lymphocyte subsets and monocytes from GPA patients has been described for the first time in this thesis. Lower relative quantities of mRNA encoding PD-L1 in whole PBMC, CD4+ T cells and CD14+ monocytes were observed in GPA patients on conventional therapies and GPA patients following B cell depletion therapy compared to healthy controls.

Changes in PD-L1 expression by monocytes and DCs have been observed in inflammatory and autoimmune diseases. One study has indicated that expression of PD-L1 on monocytes was deficient in patients with Crohn's disease [426]. A reduction in PD-L1 expression on peripheral CD14+ cells has been observed in patients with active Multiple Sclerosis but not stable Multiple Sclerosis [421]. In contrast, some studies have reported that the expression of PD-L1 on monocytes and DCs is up-regulated in patients in remission but not patients with active SLE [427], while another study reported an increased

proportion of PD-L1+CD14+ monocytes in both SLE patients with active disease and those in remission [428].

Animal models have identified an association between PD-L1 expression and autoimmune diseases. Studies on mice have shown that PD-L1 deficiency can cause expansion of autoreactive T cells with diabetes [279, 280].

Furthermore, studies on animal models and human samples have indicated that PD-L1 expressed by monocytes and DCs regulated Treg cell induction. Some studies indicated that PD-L1 expressed on DCs in mesenteric lymph nodes promoted the generation of antigen-specific Treg cells in a mouse model [303]. This was supported by a study on human peripheral DCs which demonstrated that blockade of PD-L1 on DCs inhibited Treg cell expansion after M. tuberculosis infection [429]. Therefore the deficiency of PD-L1 mRNA in CD14+ monocytes from GPA patients might lead to less suppression of the immune response and also cause a reduction in iTreg cell generation.

The interaction between PD-L1 on DCs and Treg cells may be bi-directional. Although TGF-β produced by Treg cells has been demonstrated to down-regulate PD-L1 expression on monocytes in SLE patients [430], Treg cells were demonstrated to promote the maturation of DCs and up-regulate PD-L1 expression on DCs which had reduced capacity to stimulate effector T cells [431]. Therefore the lower frequency of Treg cells found in GPA patients described in chapter 4 might contribute to the shortage of PD-L1 in monocytes as well.

PD-L1 has also been reported to be expressed on Treg cells [283, 284, 431]. Although some studies found that Treg cells do not suppress immune responses via PD-1/PD-L pathways [432], PD-L1 deficient Treg cells were shown to be defective in inhibiting alloreactive Th1 cells in a mouse model [283]. Moreover, a recent study illustrated that Treg cells suppress autoreactive B cell responses directly through interaction between PD-L1/PD-L2 expressed on its surface and PD-1 expressed on B cells in mice [284]. This information together implies that the reduction of Treg cells and PD-L1 mRNA in GPA patient may contribute to the pathogenesis of GPA by multiple pathways.

PD-L1 expression by CD4+ T cells may play an important role in regulating immune responses. Some animal studies have shown that PD-L1 expressed by naive T cells is required for DC maturation during microbial infection [433]. Therefore a shortage of PD-L1 mRNA in CD4+ T cells in GPA patients might result in failure of DC maturation and then lead to impaired interaction between DCs and B cells which could eventually cause autoantibody production.

Studies of animal models have shown that in addition to PD-1, PD-L1 can also interact with co-stimulatory molecule B7-1, and down regulate T cell responses [277]. Therefore the deficiency of PD-L1 in GPA patients may contribute to the failure of immune tolerance by pathways other than PD-1/PD-L1 pathway alone.

PD-L1 has been shown to be expressed on many different cell types in mouse models and has different functions. PD-L1 expression has been detected in

many organs in mouse, including thymus, spleen, cardiac endothelium and pancreatic islet [302]. PD-L1 expressed on non-hematopoietic cells has been shown to limit tissue damage during chronic virus infection in mice [434]. In a mouse model of autoimmune diabetes, PD-L1 expressed on islet cells has been demonstrated to inhibit tissue destruction mediated by CD4+ T cells [435].

PD-L1 expressed in tissue has been shown associated with human autoimmune disease and transplant tolerance as well. Increased PD-L1 expression on inflamed epithelial cells in salivary gland has been detected in patients with Sjögren's Syndrome [393]. PD-L1 expressed on brain endothelial cells has been found to inhibit T cell transmigration in patients with multiple sclerosis [299], while high expression of PD-L1 on keratinocytes has been shown to suppress allo-reactive immune responses through inducing Treg cells [436]. Therefore it would be interesting to study the expression of PD-L1 on non-hematopoietic cells, especially cardiac endothelium cells as it may contribute to the pathogenesis of vasculitis, in GPA patients in the future.

5.3.3 PD-L2 in GPA

The relative quantity of PD-L2 in whole PBMC, lymphocyte subsets and monocytes from GPA patients has been described for the first time in this thesis. Lower relative quantities of mRNA encoding PD-L2 in CD4+ T cells, CD8+ T cells

and CD14+ monocytes were observed in GPA patients on conventional therapies compared to healthy controls. The relative quantity of mRNA encoding PD-L2 in CD4+ T cells was higher in GPA patients following B cell depletion therapy than those on conventional therapies.

Like the PD-1/PD-L1 pathway, the interaction between PD-L2 and PD-1 has been reported to regulate T cell responses and affect T cell differentiation in animal models. In 2001, PD-L2 was reported to be the second ligand for PD-1, and the interaction between PD-L2 and PD-1 was shown to inhibit T cell proliferation and cytokine production in mouse models [291]. Further study indicated that like the PD-1/PD-L1 pathway, the PD-1/PD-L2 pathway also down-regulates CD4+ as well as CD8+ T cell responses in mice [247]. This was further confirmed by PD-L2 blockade in experimental autoimmune encephalomyelitis (EAE) mice, which results in T cell expansion and accelerated and more severe disease [306]. Moreover, PD-L2 was also reported to regulate the generation of inducible Treg cells in mice. One study demonstrated that PD-L2 deficient DCs induce fewer Treg cells in mesenteric lymph nodes in a mouse model [303].

The PD-1/PD-L2 pathway has been shown to regulate T cell responses in humans as well. Studies of peripheral blood from healthy human donors have illustrated that the PD-1/PD-L2 pathway down-regulates T cell proliferation and cytokine production [304, 305]. As discussed above, PD-L2 was also involved in regulating autoreactive B cell responses, as the expression of PD-L1 and PD-L2

on Treg cells were both required for inhibiting autoreactive B cell proliferation in mice [284]. Therefore the deficiency of PD-L2 mRNA in T cells and monocytes from GPA patients might result in less suppression of T cell responses, failure to suppress antoreactive B cell responses, and may contribute to the reduction of inducible Treg cells observed in chapter 4.

Although PD-1/PD-L2 interaction has been demonstrated to suppress immune responses in mouse models as well as human studies, the mechanism of inhibition has been reported to be different from that in PD-1/PD-L1 pathway. One study demonstrated that although either PD-L1 or PD-L2 deficiency resulted in EAE mouse model, PD-L1 deficient mice developed more severe disease than PD-L2 deficient mice, and inflammatory cytokines produced in lymph nodes from PD-L1 deficient mice was more than in PD-L2 deficient mice [307]. Another study reported PD-L1 and PD-L2 negatively regulated immune responses in experimental animal models of autoimmune kidney disease via different mechanisms, as different cell infiltrate and antibody deposits were observed from PD-L1 deficient and PD-L2 deficient mice [437]. Moreover, one recent study reported the molecular mechanisms of binding to PD-1 were different in PD-L1 and PD-L2 in human [438]. Therefore the deficiency of mRNA encoding PD-L1 and PD-L2 found in GPA patients might contribute to the disease pathogenesis through different pathways.

Although PD-L2 expression by un-stimulated peripheral lymphocytes was not observed in this study, expression of PD-L2 by activated cells from peripheral blood in humans has been described by others. One study indicated that PD-L2 is inducibly expressed by activated CD4+ and CD8+ T cells from human blood. An increased relative quantity of PD-L2 mRNA was also observed in activated CD4+ T cells [294]. PD-L2 expression by peripheral blood monocytes was reported to be up-regulated in multiple sclerosis patients treated with IFN-β [295]. Therefore, it would be interesting to isolate and culture lymphocytes from GPA patients, and then study the PD-L2 expression on those cells after stimulation in the future.

The expression of PD-L2 in tissues has also been described in autoimmune mouse models and human studies. One study observed PD-L2 expression on inflammatory infiltrating cells in brains from EAE mice [302]. In humans, PD-L2 was found to be expressed by macrophages and DCs in livers from patients with autoimmune liver diseases. The relative quantity of PD-L2 mRNA was observed to be higher as well in livers from patients with autoimmune liver diseases compared to normal controls [296]. In addition, another study demonstrated that PD-L2 is expressed by renal tubular epithelial cells in the kidneys from patients with lupus nephritis [297]. Therefore it would be interesting to study the expression of PD-L2 in inflammatory tissues in GPA patients in the future.

5.3.4 VISTA in GPA

The relative quantity of VISTA in whole PBMC, lymphocyte subsets and monocytes from GPA patients has been described for the first time in this thesis. No significant difference in the relative quantity of mRNA encoding VISTA was found in all cell types studied.

VISTA has been reported to be homologous to PD-L1 while not interacting with PD-1 in mice. It has also been shown to negatively regulate T cell-mediated immune responses in EAE mice [311]. Different from B cells from spleens in the mouse, a small amount of mRNA encoding VISTA was observed in human peripheral blood. However, no difference in the relative quantities of VISTA mRNA was found in whole PBMC, different lymphocyte subsets, or monocytes from GPA patients compared to healthy controls. There is therefore no evidence to suggest that VISTA is involved in the pathogenesis of GPA.

5.3.5 PD-1, PD-L1, PD-L2 and VISTA in SLE

Significantly lower relative quantity of mRNA encoding PD-L1 in CD4+ T cells, but not other cell types, was observed in patients with SLE compared to healthy controls. No significant difference in the relative quantities of mRNA encoding PD-1, PD-L2 and VISTA was found in patients with SLE compared to healthy controls.

Previous studies have shown a reduction in the percentage of PD-1 expressing CD4+ T cells and CD8+ T cells, with an increase in the proportion of PD-L1 expressing monocytes in SLE patients [418, 427, 428]. However, no difference in either proportion of PD-1+ CD4+ T cells or mRNA encoding PD-1 was found in any lymphocyte subsets or monocytes from SLE patients in our study. This may be because of either the limited sample numbers involved in this study or the lack of correlation between encoding mRNA level and protein expressed. In our study, a reduction in mRNA encoding PD-L1 in CD4+ T cells, while no difference in monocytes was found in SLE patients. This may be because SLE is a disease with various symptoms and a range of subtypes. Patients involved in our study were not sub-divided since they were used as disease controls for the study of GPA. It would be interesting to study the expression of PD-1 and its ligands PD-L1 and PD-L2 by lymphocytes and monocytes from well defined subtypes of SLE patients in the future.

5.3.6 Conclusion

- Although no difference in mRNA encoding PD-1 was observed in peripheral blood lymphocytes and monocytes from GPA patients, PD-1 may still be involved in the pathogenesis of GPA through reduced PD-1/PD-Ls signals.
- The shortage of mRNA encoding PD-L1 in circulating CD4+ T cells and CD14+monocytes from GPA patients may contribute to the disease pathogenesis in multiple pathways.
- The deficiency of mRNA encoding PD-L2 in peripheral CD4+ T cells, CD8+ T cells and CD14+ monocytes from GPA patients may also contribute to the disease pathogenesis.
- No difference in mRNA encoding VISTA in circulating lymphocyte subsets and monocytes from GPA patients was observed implies that VISTA is not involved in the pathogenesis of GPA.

Chapter 6

Overview and potential future directions

The aim of this thesis was to investigate the immunology in GPA and to increase understanding of this disease, as immunological features of GPA are not well studied.

In chapter 3 of this thesis, chronic activated B cells surrounded by abundant B cell survival factors and autoantibody target neutrophils without the presence of organized lymphoid structure were observed in lesions from GPA patients. This suggests that B cells in lesions from GPA patients could be stimulated by sustained PR3 expression, and their survival could be supported by local BAFF and APRIL production. In addition, B cells in the lesion might not be a component of any circulating B cell subsets that could migrate to the target tissue, as no evidence for this was observed in the study of B cell clonality. This suggests that B cell responses and autoantibody production in lesions from GPA patients is independent of circulating B cells. Once B cells migrate to the lesion, they may be supported by abundant local BAFF and APRIL to survive.

No difference in proportions of different circulating B cell subsets that might have the potential to migrate to lesions was found in GPA patients compared to healthy controls. This suggests that unlike B cells in lesions, circulating B cells in peripheral blood from GPA might not contribute directly to the pathogenesis of GPA. Later studies of circulating T_{FH} cells and Treg cells in peripheral blood from GPA patients on conventional therapies and GPA following BCD therapy suggests that B cells might contribute to the pathogenesis of GPA through

affecting frequencies of circulating T_{FH} cells and Treg cells. Altering frequencies of such immunomodulatory T cell subsets may break the balance between up-regulating and down-regulating inflammation in GPA.

In chapter 4 of this thesis, higher frequency of circulating T_{FH} cells while lower frequency of Treg cells was observed in peripheral blood from GPA patients on conventional therapies compared to controls. However, BCD treated GPA patients were no different to healthy controls in the frequencies of these T cell subsets. This suggests that the frequencies of circulating T_{FH} cells and Treg cells in peripheral blood might be B cell dependent, and the changes found in GPA patients might contribute to the pathogenesis of GPA.

The increased proportion of circulating T_{FH} cells in peripheral blood from GPA patients might lower the threshold for autoreactive B cells to survive. In addition, although no organized lymphoid structure was found in most lesions from GPA patients, PD-1+ T cells were observed in the lesions mixed with B cell infiltrates. These cells could have the functional capacity of T_{FH} cells and may provide help to local autoreactive B cells.

Although the frequencies of Treg cells in peripheral blood from GPA patients on conventional therapies were found to be decreased, the suppressive capacity per Treg cell was found to be the same as it in healthy controls. This suggests that the failure to control autoimmune responses in GPA might partly be due to changes in frequency of Treg rather than functional deficiency. The pathogenic

pathway through Treg and T_{FH} cells in GPA and the possible explanation for how B cell depletion therapy works in GPA is hypothesised in figure 6.1.

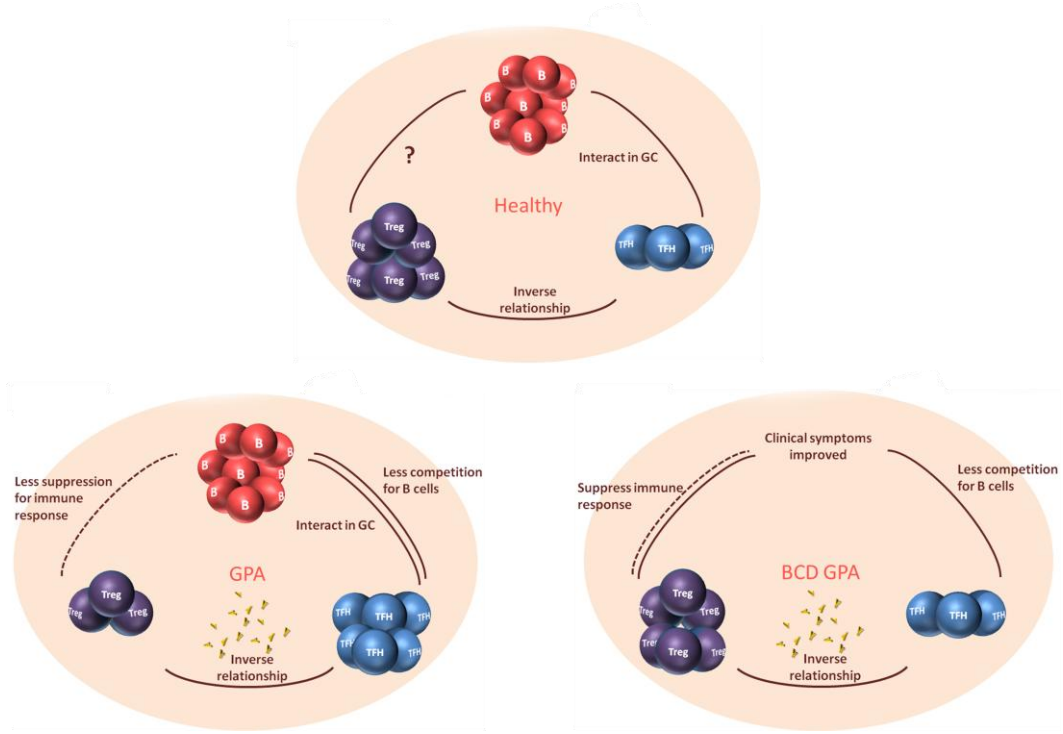

Figure 6.1 Hypothesis of pathogenic pathway through Treg and T_{FH} cells and the possible mechanism of B cell depletion therapy in GPA. **Top:** In healthy individuals, T_{FH} cells interact with B cells in GCs, provide help for B cell survival and Ab production. The frequencies of Treg cells and cT_{FH} cells in peripheral blood are inversely correlated. The functional link between Treg cells and B cells is not clear yet. **Bottom Left:** In GPA patients, there are more T_{FH} cells which provide more help to B cells and result in less competition for B cells producing low affinity Abs to survive. Fewer Treg cells in GPA patients will lead less suppression to immune responses. Therefore B cells in GPA patients could produce autoantibodies and release them to peripheral blood. **Bottom Right:** When GPA patients are treated with rituximab, B cells are depleted; the frequencies of T_{FH} cells reduce, while Treg frequencies increase to normal level which provides suppression for immune responses. Therefore the patients are clinically improved.

As the frequencies of circulating T_{FH} cells and Treg cells were inversely correlated in human peripheral blood, in the future it could be possible to

longitudinally investigate these changes in GPA patients before and after receiving BCD treatment to see whether this ratio changes in a single patient during treatment and determine if this could be developed as a biomarker for disease relapse in GPA. This study could also attempt to link the frequencies of circulating T_{FH} and Treg cells to the BVAS disease activity score.

The reasons for the increased frequencies of circulating T_{FH} cells and decreased frequencies of Treg cells in peripheral blood from GPA patients are not clear. Likewise, the mechanism of dependence of frequencies of circulating T_{FH} cells and Treg cells on B cells in peripheral blood is not clear. Whether it is affected by B cells directly or through other mediating molecules is not known. The study of PD-1/PD-Ls in chapter 5 of this thesis might provide clues to enable to address these questions.

In chapter 5 of this thesis, decreased levels of mRNA encoding PD-L1 and PD-L2 were found in circulating T cells and monocytes from GPA patients compared to healthy controls. Therefore, although no difference in mRNA encoding PD-1 was observed in peripheral blood lymphocytes and monocytes from GPA patients, PD-1 may still be involved in the pathogenesis of GPA through reduced activity of PD-1/PD-L pathways.

Previous studies have reported that PD-L1 can promote Treg differentiation while inhibiting T_{FH} cell development through interacting with PD-1 expressed on antigen presenting cells [166, 282]. Therefore, increased frequencies of

circulating T_{FH} cells and decreased frequencies of Treg cells found in peripheral blood from GPA patients might due to a deficiency of PD-L1 protein on T cells and monocytes. This could be further investigated in the future. As all data obtained in this thesis is generated from freshly isolated cells, future study could measure expression of and manipulation of PD-1 and PD-Ls in response to stimulation in vitro. The hypothesis of abnormal frequencies of Treg and cT_{FH} cells caused by deficiency of PD-L1 in GPA patients is shown in Figure 6.2.

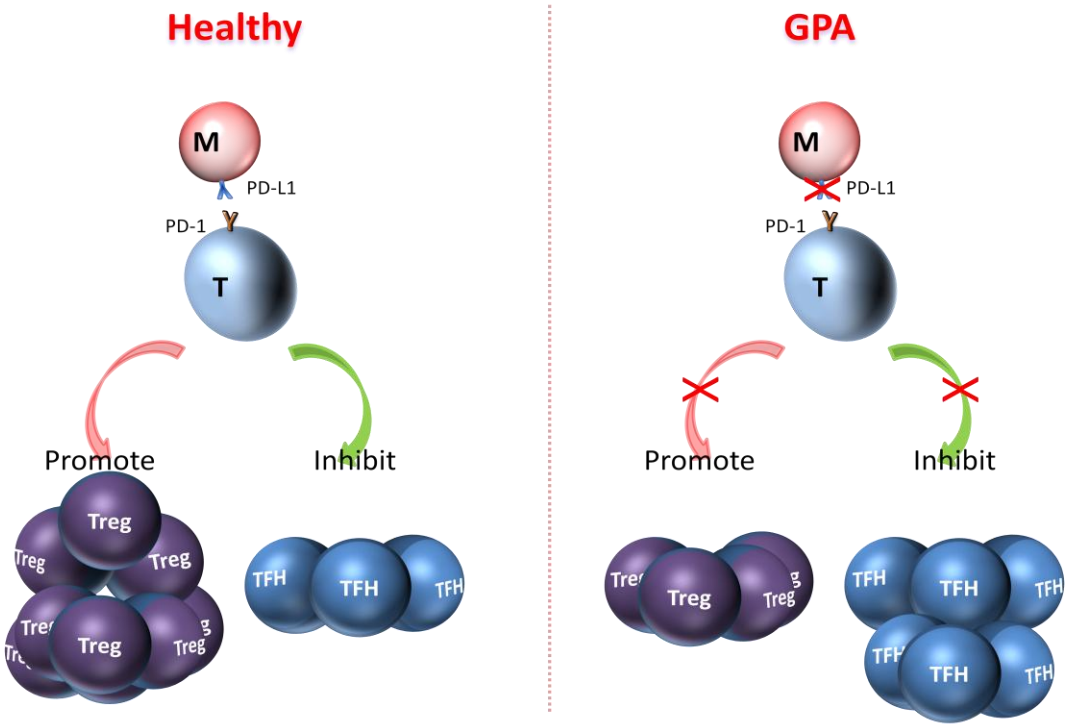

Figure 6.2 Hypothesis of PD-L1 deficiency affects Treg and cTFH cell frequencies in GPA patients. Signal through PD-L1 and PD-1 promotes Treg cell differentiation and inhibits T_{FH} cell development in healthy people. While in GPA patients, the deficiency of PD-L1 on monocytes would lead to failure of promoting Treg and inhibiting T_{FH} developments which results in less Treg cells and more T_{FH} cells in GPA patients.

Time and funding did not permit these experiments, but the data generated in this thesis has identified a number of pathways that appear highly relevant to the pathogenesis of GPA, which could be investigated in the future.

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Granulomatosis with polyangiitis involves sustained mucosal inflammation that is rich in B-cell survival factors and autoantigen

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Abstract

Objective. Granulomatosis with polyangiitis (GPA) is a rare chronic autoimmune disease that may be triggered by upper airway infection. ANCAs specific for PR3 that is expressed by activated neutrophils and macrophages are associated with GPA. Our aim was to investigate regional immune mechanisms that might induce or support the autoimmune response in GPA.

Methods. Biopsy samples from 77 patients including 8 with GPA were studied by immunohistochemistry. B-cell homing subsets in blood samples from 16 patients with GPA and 11 healthy controls were studied by FACS. The distribution of B-cell clones was searched in paired biopsies and blood samples from one patient by analysing immunoglobulin heavy chain gene (IGH) junctional sequences.

Results. Activated B cells were located alongside PR3-expressing cells and B-cell survival factors BAFF and APRIL in mucosa from patients with GPA. We detected APRIL production by the granulomas and giant cells. B cells were proliferating in all cases and persistent for 5 years in biopsies obtained from one patient. However, there was no evidence of B-cell clones from the mucosal biopsies circulating in peripheral blood in GPA or any numerical or proportional change in B-cell subsets expressing markers of regional homing in blood in GPA.

Conclusions. Our study illustrates chronically activated B cells alongside autoantigens and B-cell survival factors in the mucosa in GPA.

Key words: granulomatosis with polyangiitis, B cells, granuloma, APRIL, immunoglobulin genes.

Introduction

Granulomatosis with polyangiitis (GPA) is a rare, multi-organ inflammatory disease that may affect lungs, bronchi, oral or nasal mucosa, eyes, kidneys, nerves, brain, joints and skin [1, 2]. GPA is associated with the production of autoantibodies to neutrophil cytoplasmic antigens (ANCAs) in particular antibodies to the proteinase 3 (PR3) autoantigen expressed by neutrophils and activated macrophages [3–6]. Friederich Wegener first observed that patients with this disorder almost universally had upper airway disease especially affecting the facial sinuses, and he proposed that the disease was initiated in the upper airways [7, 8]. It has been suggested that *Staphylococcus aureus* infection may be associated with the pathogenesis of GPA and persistent *S. aureus* nasal carriage is associated with a risk of disease relapse [9, 10]. The chronological sequence of upper airway symptoms followed by systemic manifestations, including a high incidence of thromboembolic events [11], is consistent with the notion that GPA may evolve as a consequence of upper airway inflammation and that other systemic, potentially life-threatening symptoms are a

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direct consequence of the inflammation and activity of pathogenic autoantibodies.

The immunological features of the mucosal microenvironment in GPA have not been studied extensively. Classical features of the disease include a mixed infiltration of acute and chronic inflammatory cells, vasculitis, granulomas and small numbers of giant cells [12–14]. It has been shown that B cells are present in the nasal mucosa in GPA and that the immunoglobulins they express are encoded by genes that are mutated in their immunoglobulin variable regions, a feature of germinal centre (GC) origin [15, 16]. Despite this, conventional germinal centres have not been identified as a consistent feature of the inflamed airway mucosa in GPA, though GC-like structures have been described [17]. B cells in normal oro-nasal mucosa and bronchial mucosa have been identified in organized lymphoid structures termed nasal-associated lymphoid tissue (NALT) or bronchus-associated lymphoid tissue (BALT), respectively [18]. In addition, isolated activated B cells can be identified beneath the stratified/pseudostratified epithelium in oral mucosa that are increased in frequency and are associated with plasma cell differentiation in local inflammatory responses [19].

In this study, we have characterized the B-cell infiltrate in mucosa in GPA. We have related the distribution of B cells to the distribution of cells expressing or binding the PR3 autoantigen and to the local production of B-cell survival factors, B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) by immunohistochemistry. We observe that large activated B cells in GPA exist alongside an abundance of cells expressing PR3. The B cells in GPA mucosa express BAFF receptor and are therefore potentially able to receive survival signals from locally produced BAFF. APRIL is expressed abundantly by many cell types in GPA mucosa, but most notably by the granulomas and giant cells that are a feature of the disease.

Immunoglobulin heavy chain gene (IGH) analysis allows the identification of B-cell clonality by comparison of junctional sequences that are unique to each B cell and its clonal progeny. By immunoglobulin variable region gene analysis, we observed evidence for local and sustained B-cell clones in the upper bronchial mucosa in GPA in a single case studied. We also studied subsets of blood lymphocytes and saw no evidence for preferential expansion of cells associated with any anatomically defined circulation pathways. By IGH analysis, there was no evidence of the B-cell clones identified in the mucosa circulating in the blood.

Overall, our study supports the concept that the mucosa in GPA is a niche containing chronically activated B cells and autoantigen. A local autoantibody response as described previously [20] could be sustained by the abundance of local B-cell survival factors.

Materials and methods

Tissues

Paraffin blocks of inflamed mucosal biopsies from 77 patients, including 8 patients with clinically confirmed GPA, were selected to include a range of characteristics from mild lymphocytic infiltrates considered within normal histological limits through to marked inflammation (detailed location of the tissue sampled and the diagnoses are included in supplementary Table S1, available as supplementary data at *Rheumatology* Online). The group of patients with orofacial granulomatosis (OFG) were considered to be an important control group because this is a chronic granulomatous mucosal lesion, but it is not associated with acute inflammation or autoimmunity. Specimens were graded according to the inflammatory infiltrate present to produce a semi-quantitative score (Nil, Mild and Marked). This was undertaken blind by two authors (P.P. and E.O.).

In addition, peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Paque from fresh blood samples donated by 16 patients with GPA (M:F ratio 1:1, age between 25 and 73 years, average age 53 years) and 11 healthy controls (M:F ratio 1:1, age between 22 and 68 years, average 45 years). The details of patients' clinical information are listed in supplementary Table S2, available as supplementary data at *Rheumatology* Online.

Flow cytometry (FACS)

PBMCs were isolated and incubated with fluorochrome-conjugated mAbs on ice for 40 min. To isolate Beta7^{low} CLA+ CCR10− B cells, PBMCs were stained with CD19-PE (BD Biosciences 10 μl/100 μl), Beta7-PeCy5 (BD Biosciences 10 μl/100 μl), CLA-FITC (Biolegend, 15 μl/100 μl), CCR10-APC (R&D Systems, 7 μl/100 μl). After staining, cells were washed and resuspended in sterile PBS and immediately separated into different subpopulations with the BD FACSAria I cell sorter or BD FACSCanto.

Immunohistochemistry

Paraffin-embedded tissue sections (5 μm) were submitted to antigen retrieval by heating retrieval solution (DAKO, Glostrup, Denmark) at 95°C for 40 min. Endogenous peroxidase and alkaline phosphatase were blocked. Sections were single stained for CD20, APRIL, BAFF, BAFF-R, PD1, CD57, IgD, IgG, IgA, IgM and IgE and double stained for CD138 with CD20, Ki67 with CD20, neutrophil elastase with CD20, PR3 with CD20, PR3 with neutrophil elastase and E-selectin with CLA using the EnVision single and double stain systems (DAKO). After staining, sections were counterstained with haematoxylin.

Analysis of immunoglobulin gene sequences

IGH from blood cells was amplified from the variable (IGHV) to constant (IGHC) regions. IGH from paraffin-embedded biopsy sections was amplified from the framework region 3 (FR3) to the joining (J) region. Semi-nested PCR was applied (details in supplementary Table S3, available as supplementary data at *Rheumatology* Online). To search the clone relationships between biopsies and different circulating blood subsets, clone-specific primers were applied to all blood subsets and biopsies from the same patient using the FR3-IGHJ PCR program.

All primers used are listed in supplementary Table S4, available as supplementary data at *Rheumatology* Online.

Ethics approval

This study was approved by the National Research Ethics Service (Approval no. 10/H0715/3). All patients donated blood and agreed to allow access to biopsy and tissue samples that were surplus to clinical investigation after fully informed written consent.

Statistical methods

Data sets are expressed with average values and comparisons were made using the Mann–Whitney test. Statistical analysis was carried out using GraphPad Prism4.

Results

B cells and plasma cells as components of chronic/acute inflammation in GPA

B and T cells were consistently present in mucosal biopsies from eight patients with GPA. B cells in most cases tended not to be components of any organized lymphoid structure but were scattered submucosally, and the infiltrate showed no evidence of GC formation. One nasal biopsy from a patient with GPA contained abundant organized mucosa-associated lymphoid tissue (MALT), but the affected mucosa in the remaining cases had no evidence of GC formation. The B cells in GPA had properties of activated cells; they did not express IgD but were large cells with extensive dendritic processes that extended and made contact with many adjacent cell types. Approximately 5% expressed the Ki67 nuclear proliferation antigen. This description of B-cell morphology, phenotype and proliferative behaviour does not fit easily within the current schemes of B-cell biology. These cells were therefore compared with those seen in a range of inflammatory conditions of different levels of severity involving mucosal surfaces, including those around the oral cavity and upper airways. This B-cell subset was indistinguishable from that consistently seen in other biopsies of nasal and oral mucosa stained as controls, demonstrating that the presence of this B-cell subset was not a disease-specific observation. Unlike the controls, used in this study, however, the B-cell infiltrate in GPA was generally intimately mixed with acute inflammatory cells that included cells expressing the GPA-associated autoantigen PR3 and neutrophil elastase.

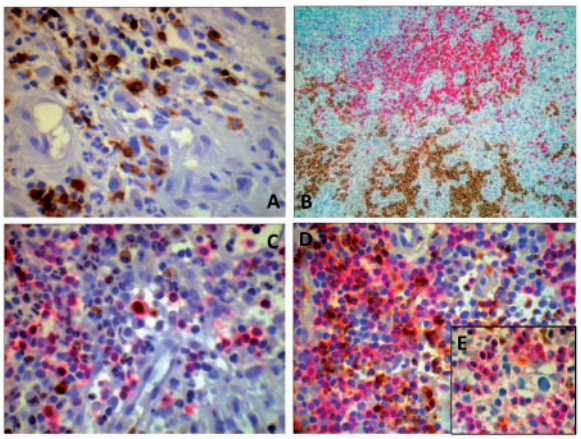
The plasma cell and B-cell infiltrates tended to occupy adjacent areas of mucosa. Plasma cells were often observed in the infiltrate that was predominantly B cells, but B cells were rare in the plasma cell-dominated zones. The majority of plasma cells expressed IgG rather than IgM or IgA, consistent with the profile observed in the control samples (Fig. 1).

Analysis of local B-cell clonality by Ig gene analysis

DNA was prepared from serial sections of diagnostic biopsies from one patient (patient P5 in supplementary Table S2, available as supplementary data at *Rheumatology* Online) who donated surplus tissue following surgical procedures in 2004, 2009 and 2010 and a blood sample. Ten PCRs to amplify IGH from FR3 to J were carried out from each of the biopsies. Clones with the same junctional sequence from the same PCR were considered to be the same sequence and were counted as one. A total of 74 different rearrangements were identified; 40 from bronchial mucosa in 2004, 13 from bronchial tissue in 2009 and 21 from nasal mucosa in 2010. The different numbers were a reflection of different B-cell numbers in the different-sized tissue samples studied. Clone-specific primers were then prepared to the V proximal N-D sequence to specifically identify the clone in the target DNA sample by PCR. Design ensured that some non-templated sequence was present between the clone-specific primer and the J segment primer to allow confirmation of clonal identity by sequencing. Of 14 primers designed, 8 were found not to amplify non-specifically in a DNA preparation of tonsil cells from an unrelated individual and were considered to be potentially clone specific. When PCR products of the correct size were generated by clone-specific PCR, clonal identity was confirmed by sequencing.

Although the B-cell population was polyclonal, these methods identified three examples of B-cell clonal

Fig. 1 Examples of B lineage cells in paraffin sections of GPA mucosa studied by immunohistochemistry.



(**A**) Single staining with CD20 (brown) identified B cells in biopsies from GPA patients. (**B**) Double staining with CD20 (pink) and CD138 (brown) identified B cells and plasma cells occupying adjacent areas in GPA. Plasma cells were present in the B-cell-dominated areas while B cells did not tend to infiltrate plasma cell-dominated areas. (**C**) Double staining of cell division antigen Ki67 (brown nucleus) and surface CD20 (in pink) identified B cells that were proliferating in the biopsies. (**D**) Double staining of CD20 (pink) and neutrophil elastase (brown) identified that activated B cells were adjacent to neutrophils, the target of autoantibody ANCA, in GPA biopsies. (**E**) Double staining of PR3 (in brown), the main target of ANCA in GPA, and CD20 (pink).

expansion in the inflamed mucosa. Two examples were restricted to the 2009 biopsy. Remarkably, one clone was observed to be present in bronchial tissue in both 2004 and 2009 (Fig. 2). The same method was also applied to detect circulating B-cell clones in peripheral blood. However, we did not observe any evidence that B-cell clones in the lesion were circulating in peripheral blood.

Local production of B-cell survival factors in GPA

Since we saw evidence of local B-cell division and sustained clonal expansion as described above, we investigated the local distribution of B-cell survival factors APRIL and BAFF and the BAFF receptor [21–24], all of which can be detected by immunohistochemistry in paraffin-embedded tissue. APRIL was produced abundantly in the B-cell microenvironment in GPA and in other control mucosal biopsies. It was observed that granulomas and giant cells, both of which are features of most cases of GPA, contained abundant APRIL. Granulomas in OFG and sarcoid also contained APRIL. Granulomas are, therefore, a previously unrecognized source of APRIL that may contribute to the support of the local B-cell response. The B cells in GPA expressed the BAFF receptor, and cells producing BAFF were also identified in the mucosa, indicating that this pathway of promoting B-cell survival is also relevant to the local response in GPA (Fig. 3).

Analysis of lymphocytes expressing regional homing receptors in GPA

Evidence presented above is consistent with a local and sustained B-cell response in the mucosa in GPA. We investigated whether the chronic expansion of B cells in the mucosa was associated with any changes in the proportion of total lymphocytes or B cells in blood, which express molecules that facilitate regional lymphocyte homing. Cells that home to the nasal and bronchial mucosa have been reported to express the cutaneous lymphocyte antigen (CLA), which binds the endothelial antigen E-selectin, but not the chemokine receptor CCR10 [25–28].

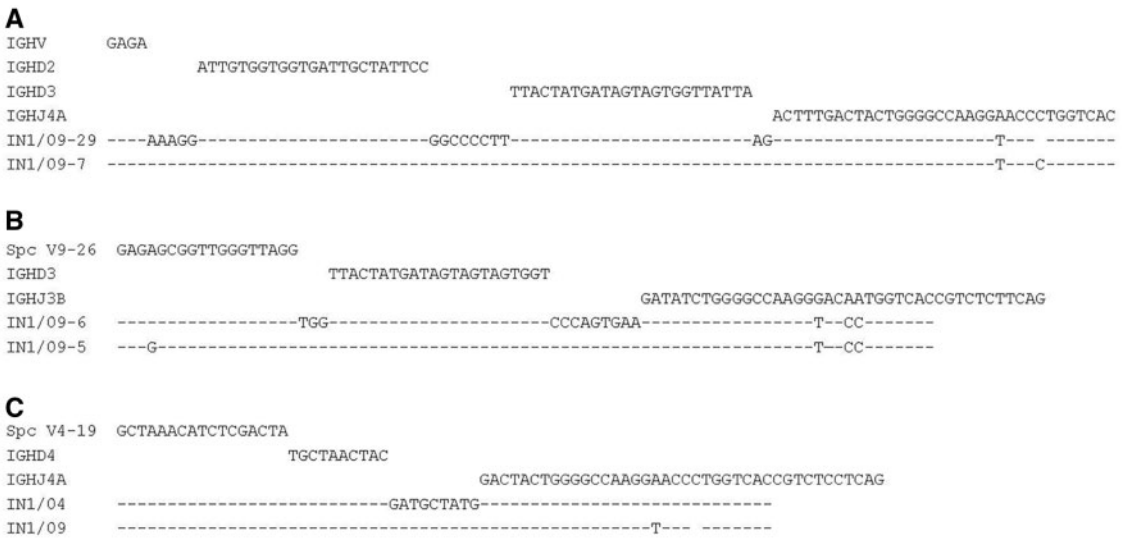
We confirmed the expression of E-selectin by flat endothelium in mucosal biopsies in GPA. We then identified cells expressing CLA, the receptor for E-selectin, either among B cells or in the total lymphocyte gate by flow cytometry. There was no difference between patients with GPA and healthy controls in the proportion of B cells or lymphocytes expressing CLA or any other lymphocyte subset associated with regional homing (Fig. 4).

The clone-specific PCR method as described above was applied to the B-cell subsets isolated from blood, using primers designed to the CDR3 sequences observed in the biopsy taken from the patient at the same time. No evidence of the B-cell clones identified in the affected mucosa was observed in the blood in any sorted subsets.

Discussion

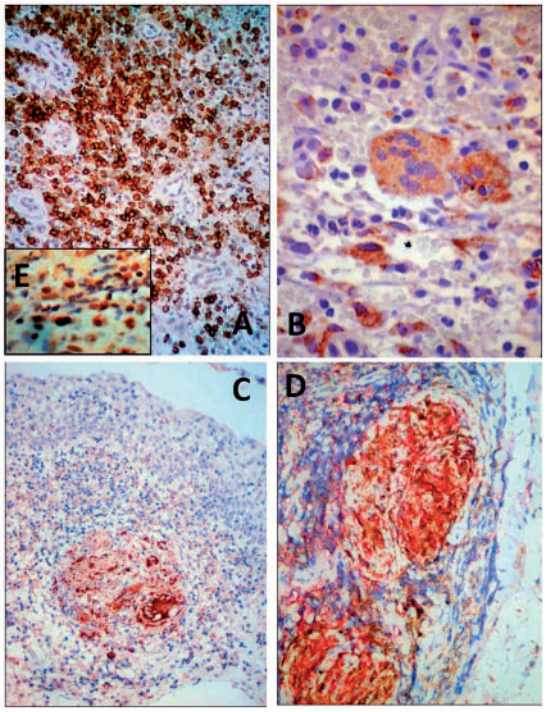
In this study we observed that inflamed mucosa in GPA contains chronically activated B cells intimately mixed with cells expressing PR3, a characteristic target of autoantibodies in most cases of GPA. The mucosal microenvironment is rich in B-cell survival factors APRIL and BAFF and the expression of APRIL by granulomas and giant cells is described here for the first time. The

Fig. 2 IGH VDJ junction sequences from a patient with GPA.



(**A**) and (**B**) are alignments of sequences of VDJ obtained from different DNA samples of a biopsy obtained in 2004. The presence of the same sequence in different PCRs confirms B-cell division and local presence of members of a B-cell clone. (**C**) One VDJ rearrangement was identified in both the 2004 and the 2009 biopsies from this patient, implying that this B-cell clone was persistent in the lesion for at least 5 years.

Fig. 3 Paraffin-embedded biopsies studied by immunohistochemistry to identify local B-cell survival factors.



(**A**) Single staining of BAFF-R (in brown) identified the distribution of B cells that expressed receptor for survival factor BAFF in biopsies from GPA patients. (**B**) Single staining of APRIL (in brown) illustrated the B-cell survival factor APRIL production in GPA. Giant cells producing APRIL were observed in GPA. (**C**) Single staining of APRIL (in brown) in OFG. A granuloma containing a giant cell producing APRIL is shown here. (**D**) Double staining of APRIL (brown) and CD68 (pink) illustrated the APRIL was produced by the macrophages that comprise the granulomas in sarcoidosis. (**E**) Single staining of BAFF (in brown) identified the production of B-cell survival factor BAFF in GPA.

combination of mixed acute and activated chronic inflammatory cells, alongside survival factors including APRIL produced by granulomas and giant cells, were only seen in GPA.

The B cells observed in GPA were indistinguishable as a population from recently identified subepithelial dendritic B cells in oral mucosa [19]. In GPA and a varied panel of controls, B cells were activated cells that lack IgD, contain a small but consistent proliferating fraction and have irregular cytoplasmic processes that extend and contact diverse adjacent cells. They are not associated with any follicular structures but are present in increased numbers and exist alongside a plasma cell component in inflammation. Such cells are not a general feature of the physiological process of plasma cell differentiation from B cells. For example, the lamina propria of the normal gut, a site of plasma cell differentiation, does not have a significant population of cells that resembles those observed in the mucosa described here. In GPA, the most common Ig isotype observed was IgG, consistent with the profile observed in the diverse set of control tissues.

Local B-cell division observed by immunohistochemistry was supported by the detection of clonally related cells in a single bronchial biopsy studied by Ig gene analysis. Clonally related cells were detected in a polyclonal B-cell background by comparison of CDR3 sequences. The presence of a clonal population of B cells persisting for 5 years in one patient suggests that B-cell stimulation is chronic.

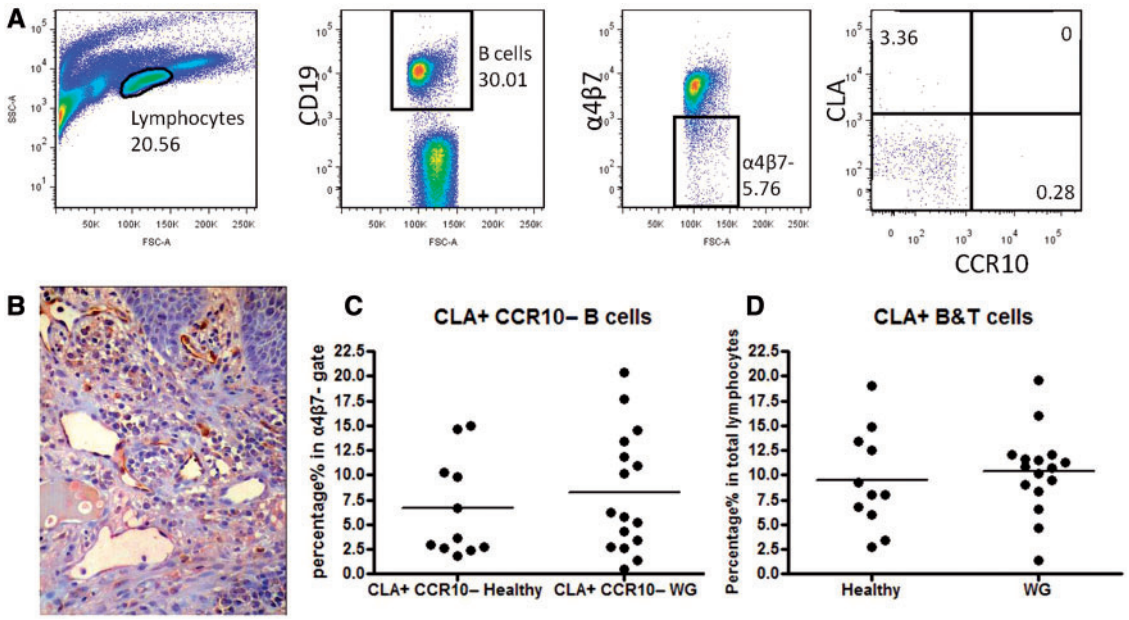
In GPA, the B cells were often intimately associated with cells expressing PR3. Some PR3-positive cells were clearly neutrophils, but not all. It is possible that the abundance of PR3 antigen locally provides an immunological stimulus to potentially initiate or sustain the chronic B-cell anti-PR3 response. However, some cases studied were negative for autoantibodies to PR3. This demonstrates that while this juxtaposition could possibly support the generation of autoantigen reactive B cells, this does not necessarily occur.

B-cell responses that result in GC formation are generally T-cell dependent [29]. T-cell involvement places stringent regulation on the discrimination between self and non-self as a consequence of thymic education [30, 31]. The chronic B-cell activity but lack of GC formation in most disease-relevant mucosal microenvironments in GPA might indicate that the anti-PR3 response in the mucosa could be driven in a T-cell independent way.

B-cell survival factors APRIL and BAFF were present in the nasal mucosa in GPA. We observed for the first time in this study that granulomas and giant cells are both significant potential sources of APRIL. It is possible that the granulomas, that are also linked to macrophage fusion and giant cell formation, may subsequently provide a source of factors to support an aberrant B-cell response. This observation is relevant to granulomatous diseases other than GPA and is therefore not restricted to this autoimmune condition. Studies have not observed correlation between concentrations of APRIL in serum and ANCA titres [32], and the relevance of serum BAFF to ANCA status and vasculitis remains unclear in a study of GPA serum BAFF varied inversely with ANCA levels [32–34]. It is not known if BAFF and APRIL produced in mucosa acts locally or if it may contribute to the serum pool of B-cell survival factors.

The B cells in mucosal lesions in GPA have been observed to have mutated IGHV genes, yet in the majority of cases no GC formation was observed in the mucosa [15, 16]. We therefore considered that the B-cell fraction that localizes to the mucosa might be expanded as a population in blood in GPA as part of a migratory continuum. It is also possible that recruitment to an inflammatory site might result in depletion of a subset of inflammatory cells from the blood. When we analysed the subsets of total lymphocytes or B cells in blood that expressed markers associated with tissue-specific

FIG. 4 Analysis of blood lymphocytes expressing regional homing receptors in GPA.



(**A**) Flow cytometry profiles for CLA expression to identify B cells that migrate to nasal and upper airway mucosa. PBMCs from patients with GPA and healthy controls were stained with CD19, then α4β7, CLA and CCR10. (**B**) Imunohistochemical staining of E-selectin (in brown), which is the endothelial receptor for CLA in GPA. (**C**) Percentages of α4β7−, CLA+ CCR10− B cells in 16 patients with GPA and 11 healthy controls ($P = 0.57$ by Mann–Whitney analysis). (**D**) Percentages of CLA+ cells (including B cells and T cells) of total lymphocytes in 16 patients with GPA and 11 healthy controls ($P = 0.54$ by Mann–Whitney analysis). Average values are marked by lines on the scatter plots.

homing, no differences between GPA and healthy controls were apparent.

Overall our data are consistent with the hypothesis that a chronic, local B-cell response in inflamed mucosa, in the presence of abundant PR3 antigen and supported by locally produced B-cell survival factors BAFF and APRIL, could support the production of the autoantibodies that are associated with life-threatening systemic symptoms in GPA.

Rheumatology key messages

- B cells in the mucosa in GPA are mostly isolated, activated cells.
- In a single case studied, local mucosal B cells included long-lived clones.
- B-cell survival factor APRIL produced by granulomas could promote B-cell survival in granulomatous lesions.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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